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Diet-induced obesity (DIO) is linked to several adverse neurobiological and behavioral changes, including altered trace element homeostasis, dysregulated dopamine biology, increased anxiety, and reduced physical activity. These DIO-associated harmful effects on the brain can be influenced by sex and strain. The studies presented in this dissertation address both main effects and interactions between diet, sex, and strain on the obesity-induced dysregulation of iron, manganese, copper, and zinc status in the brain, dopamine release and clearance, behavior related to physical activity, anxiety, motivation, and memory, and mRNA expression of genes related to trace element homeostasis, behavior, and neurodegenerative disease. Male and female C57BL/6J (B6J) and DBA/2J (D2J) mice were fed either a low fat diet (LFD) with 10% kcal from fat or a high fat diet (HFD) with 60% kcal from fat for 16 weeks. Our studies revealed a heterogeneous effect of DIO on many of the neurobiological variables that we evaluated. For example, in the striatum, iron was significantly elevated in B6J female mice but not male mice due to DIO. Similarly, in the hippocampus, zinc was increased in D2J males but decreased in D2J females. There was also a dramatic induction of divalent metal transporter 1, alpha synuclein, and amyloid precursor protein in this brain region due to DIO, but only in the B6J males. Behavior assessments demonstrated that B6J male mice fed a HFD were impacted the most through their display of significantly

reduced locomotion, reduced rate of habituation, lack of motivation, and elevated anxiety levels. Interestingly, these mice also showed a significant upregulation of dopamine receptor D2. Dopamine clearance in the dorsal striatum was significantly reduced in both male and female D2J mice due to DIO, while in the nucleus accumbens core, reductions in dopamine clearance occurred for male mice of both strains fed HFD. Collectively, these data provide evidence for important sex and strain differences on the impact of DIO-associated behavior alterations and neurobiology dysregulation. As the incidence of obesity continues to rise worldwide, these findings have key health implications related to debilitating behavior disorders and the development of neurodegenerative disease that can be triggered by an energy dense diet and a state of DIO.

THE INFLUENCE OF SEX AND STRAIN ON NEUROBIOLOGICAL AND BEHAVIORAL
CHANGES DUE TO DIET-INDUCED OBESITY

by

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TABLE OF CONTENTS

	Page
LIST OF TABLES.....	ix
LIST OF FIGURES.....	x
LIST OF ABBREVIATIONS IN FIGURE TITLES.....	xii
 CHAPTER	
I. INTRODUCTION	1
II. LITERATURE REVIEW	5
Significance	5
Trace Element Dysregulation in the Brain	6
DIO Impact on Iron and Gene Expression in the Brain	7
Sex as a Biological Factor	8
Genetics as a Biological Factor.....	9
Combined Sex and Genetic Factors	9
Behavior and DIO	10
DIO and Physical Activity	11
DIO and Anxiety	12
DIO and Memory.....	14
DIO and Dopamine.....	15
Sex Factors in Murine Behavior	16
Gene Expression Dysregulation in the Brain	17
Divalent Metal Transporter 1	17
Iron Regulatory Protein.....	20
Ceruloplasmin	21
Copper Transporter 1.....	24
Alpha Synuclein	25
Amyloid Precursor Protein.....	29
Brain-Derived Neurotrophic Factor	31
Tyrosine Hydroxylase	34
Dopamine Receptor D2.....	36
Conclusion.....	38

III. THE INFLUENCE OF SEX AND STRAIN ON TRACE ELEMENT DYSREGULATION AND GENE EXPRESSION ALTERATIONS IN THE BRAIN DUE TO DIET-INDUCED OBESITY	39
Abstract.....	39
Introduction	40
Materials and Methods.....	44
Animals and Diet	44
Tissue Collection	45
Trace Element Analysis	46
RNA Isolation and cDNA Synthesis	46
Real Time Polymerase Chain Reaction (RT-PCR)	47
Statistical Analysis.....	48
Results.....	48
A HFD Causes Significant Weight Gain in Male and Female B6J and D2J Mice	48
Female Mice Fed a HFD Have Increased Fe in the Striatum.....	50
Midbrain Cu Decreases in D2J Mice Fed a HFD	51
B6J Mice Fed a HFD Have Increased Fe, Mn, Cu, and Zn in the Hippocampus, While D2J Mice Fed a HFD Show Opposite Trends in Zn Alterations.....	52
Male B6J Mice Fed a HFD Have Increased Fe and Mn in the Olfactory Bulb.....	54
The Effect of HFD on Olfactory Bulb Zn Shows Opposite Trends by Strain and Sex	55
Body Weight is Correlated to Striatal Fe and Hippocampal Zn in Females.....	56
D2J Female Mice Fed a HFD Have Increased Expression of Ceruloplasmin in the Striatum	57
DIO Significantly Impacts Gene Expression in the Hippocampus of B6J Male Mice	58
DIO Significantly Impacts Gene Expression in the Olfactory Bulb of D2J Male Mice.....	59
Body Weight is Correlated with mRNA Expression of DMT1, Alpha Synuclein, and APP in Female D2J Mice.....	61
Discussion.....	62
Effect of HFD on DIO	63
Effect of DIO in Striatum	64
Effect of DIO in Midbrain	68
Effect of DIO in Hippocampus.....	70
Effect of DIO in Olfactory Bulb.....	74

Conclusion.....	77
IV. THE IMPACT OF SEX AND STRAIN ON THE DYSREGULATION OF BEHAVIOR, DOPAMINE BIOLOGY, AND GENE EXPRESSION IN THE BRAIN DUE TO DIET-INDUCED OBESITY	79
Abstract.....	79
Introduction	80
Materials and Methods.....	88
Animals and Diet	88
Open Field	89
Novel Object Recognition	90
Nestlet Shredding	92
Tissue Collection	92
RNA Isolation and cDNA Synthesis	93
Real Time Polymerase Chain Reaction (RT-PCR)	94
Dopamine and Voltammetry	94
Statistical Analysis.....	95
Results.....	96
Mice Fed a HFD Gained a Significant Amount of Weight Compared to Mice Fed a LFD	96
B6J and D2J Mice Fed a HFD Travel Less Distance in the Open Field	99
B6J and D2J Mice Fed a HFD Have Reduced Velocity in the Open Field	101
Final Body Weight is Correlated to TDT and Velocity for Females Fed a HFD	104
Male B6J Mice Fed a HFD Have a Slower Habituation Rate	106
Male B6J Mice Fed a HFD Show Higher Anxiety-like Behavior Through Fecal Boli.....	106
DIO Did Not Impact Center Entries in the Open Field	107
Male B6J Mice Fed a HFD Have Significantly Lower Levels of Nestlet Shredding.....	107
DIO Did Not Impact Memory in NOR.....	108
Dopamine Release in the Dorsal Striatum is Increased in B6J Females Fed a HFD	109
Dopamine Reuptake in the Dorsal Striatum is Reduced for D2J Mice Fed a HFD	110
Dopamine Reuptake in the NAc Core is Reduced for Male Mice Fed a HFD.....	110

In the Striatum, DRD2 Gene Expression was Upregulated in B6J Males Due to DIO and TH Gene Expression was Significantly Higher in the B6J Strain Compared to the D2J Strain	112
In the Hippocampus, Female D2J Mice Express Significantly More BDNF Compared to D2J Males.....	113
In the Olfactory Bulb, DIO Caused an Upregulation of DRD2 and TH Gene Expression in Male D2J Mice and Induced BDNF Gene Expression in Female B6J Mice	114
Discussion.....	115
Weight Gain	116
DIO and Locomotion	116
DIO and Habituation	118
DIO and Anxiety-like Behavior	120
DIO Effect on Motivation and Welfare	122
DIO and Memory.....	123
Dopamine Release and Reuptake in the Dorsal Striatum	124
Dopamine Release and Reuptake in the NAc Core of the Ventral Striatum	125
Effect of Diet, Sex, and Strain on DRD2 mRNA Expression	127
Effect of Diet, Sex, and Strain on TH mRNA Expression	129
Effect of Diet, Sex, and Strain on BDNF mRNA Expression	130
Conclusion	132
V. EPILOGUE	135
REFERENCES	144
APPENDIX A. SUPPLEMENTARY DATA	181
APPENDIX B. BEHAVIOR TESTING	187

LIST OF TABLES

	Page
Table 3.1. Study Design Based on Stain, Sex, and Diet	45
Table 3.2. mRNA Transcripts Related to Trace Elements and Neurodegeneration	47
Table 3.3. Initial and Final Body Weight	49
Table 3.4. Correlation Between Final Body Weight and Gene Expression	62
Table 4.1. Study Design for Behavior, Gene Expression, and Voltammetry.....	89
Table 4.2. mRNA Transcripts Related to Behavior and Dopamine	94
Table 4.3. Final Body Weight	97

LIST OF FIGURES

	Page
Figure 3.1. Weight Gain by Strain	50
Figure 3.2. The Effect of HFD on Striatum Fe in B6J and D2J Male and Female Mice	51
Figure 3.3. Midbrain Cu and Mn Concentrations in B6J and D2J Mice	52
Figure 3.4 Hippocampus Trace Elements.....	53
Figure 3.5 Olfactory Bulb Iron and Manganese	54
Figure 3.6 Olfactory Bulb Zinc Three-Way Interaction	55
Figure 3.7 Final Body Weight and Trace Element Concentration Correlations	56
Figure 3.8 Gene Expression Related to Trace Element Regulation and ND	60
Figure 4.1 Weight Gain by Strain and Sex.....	98
Figure 4.2 Total Distance Traveled Sex Effect with Time.....	100
Figure 4.3 Total Distance Traveled Diet Effect with Time.....	101
Figure 4.4 Velocity Sex Effect with Time.....	102
Figure 4.5 Velocity Diet Effect with Time.....	103
Figure 4.6 Relationships between Weight and Total Distance Traveled or Velocity	105
Figure 4.7 DIO Impact on Various Behaviors in B6J and D2J Mice	108
Figure 4.8 Novel Object Recognition in Male and Female B6J and D2J Mice.....	109
Figure 4.9 Dopamine Release and Reuptake in the Striatum	111
Figure 4.10 Striatum DRD2 and TH Gene Expression	112

Figure 4.11 Influence of Sex on BDNF Gene Expression in the Hippocampus	113
Figure 4.12 DIO Impact on Olfactory Bulb DRD2, TH, and BDNF Gene Expression	115

LIST OF ABBREVIATIONS IN FIGURE TITLES

HFD = high fat diet

B6J = C57BL/6J

D2J = DBA/2J

Cu = copper

Mn = manganese

ND = neurodegenerative disease

DIO = diet-induced obesity

DRD2 = dopamine receptor D2

TH = tyrosine hydroxylase

BDNF = brain-derived neurotrophic factor

CHAPTER I

INTRODUCTION

Obesity and overweight prevalence is escalating worldwide, with an estimated 39% of adults classified as obese or overweight (Chooi et al., 2019). The adverse effects of diet-induced obesity (DIO) have been linked to neurobiological disruptions, including altered trace element homeostasis (Han et al., 2019; Liu et al., 2016), dysregulated dopamine biology (Leite and Ribeiro, 2019), and gene expression alterations (Gan et al., 2015; Huang et al., 2005; Wu et al., 2017). Furthermore, DIO is associated with negative behavioral changes such as reduced physical activity (Sanyaolu et al., 2019), increased anxiety (Baker et al., 2017), and compromised memory (Davidson et al., 2014).

Biochemical consequences of obesity include inflammation, oxidative stress, and mitochondrial dysfunction, all of which are common pathologies of neurodegenerative disease (Mazon et al., 2017). Increasing evidence implicates obesity as a risk factor for two of the most common forms of neurodegenerative disease: Alzheimer's disease and Parkinson's disease (Martin-Jiménez et al., 2017; Mazon et al., 2017). As conditions of overweight and obesity are increasing in both males and females worldwide, these alterations in neurobiology and behavior due to obesity can have serious consequences related to declining health, reduced productivity, and increased health care costs (Trogdon et al., 2008).

Sex and genetics are important biological factors to consider in the study of DIO. The inclusion of both males and females in research studies is a key initiative proposed by the National Institutes of Health (Clayton, 2018). Supporting evidence for sex differences in response to a high fat diet and a state of DIO can be found in several recent human and rodent obesity reports (Bridgewater et al., 2017; Charradi et al., 2017; Malpetti et al., 2018). Furthermore, genetic background is an important biological variable, as several recent studies indicate that strain or genetic variation can influence the effect of DIO on disease risk (Palacios et al., 2011), gene expression (Norris et al., 2016), and behavior (DeJesus et al., 2016). These human and animal studies highlight the significant influence of sex and genetics on biological alterations and eventual health outcomes that can be triggered by DIO.

Preclinical studies of DIO in rodents have provided a valuable model to elucidate the biological impact and health consequences of obesity (Barrett et al., 2016). Currently, there are very few rodent studies that address the influence of both sex and strain on DIO-associated alterations of neurobiology and behavior. To address this gap in the literature, our study design used male and female C57BL/6J (B6J) and DBA/2J (D2J) mice fed either a low fat diet with 10% kcal from fat or a high fat diet with 60% kcal from fat for 16 weeks to study the impact of DIO on the brain. These strains were selected based on their frequent use in behavioral neuroscience and prior studies exhibiting differential traits (Mozhui et al., 2010). B6J and D2J mice represent key strains in the Mouse Phenome Project Database (Bogue et al., 2018; Grubb et al., 2014)

and are used as parental strains in the BXD recombinant inbred strain set for the GeneNetwork open source project (Philip et al., 2010). Furthermore, these strains have been validated as appropriate models for DIO (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992).

Our lab seeks to understand the influence of sex and strain on neurobiological and behavioral changes induced by DIO. Our major study objectives included the following:

- 1. Identify interaction effects between diet, sex, and strain on trace element dysregulation and gene expression due to DIO in specific brain regions.** We hypothesized that the influence of sex on DIO would impact the B6J strain more than the D2J strain based on a pilot study from our lab, and trace element dysregulation would be region-specific based on our previous published studies (Han et al., 2019; Liu et al., 2016). We also hypothesized that DIO would cause increases in iron, zinc, and alpha synuclein gene expression in the olfactory bulb, as these physiological changes have been implicated in the pathogenesis of Parkinson's disease (Adler and Beach, 2016; Gardner et al., 2017). To test these hypotheses, we evaluated four trace elements (iron, manganese, copper, and zinc) and six genes for mRNA expression (divalent metal transporter 1, iron regulatory protein 1, ceruloplasmin, copper transporter 1, alpha synuclein, and amyloid precursor protein) in the following brain regions: hippocampus, midbrain, striatum, and olfactory bulb. These brain regions were selected based

on their importance to trace element neurobiology. The specific genes evaluated in our study were selected based on their role in trace element regulation in the brain and their potential connection to neurodegeneration.

2. **Investigate the impact of DIO on behavior change, gene expression, and dopamine release and reuptake using male and female B6J and D2J mice as a model to examine sex and strain influences.** We hypothesized that DIO would have a greater impact on males compared to females, and that D2J mice would be more resistant to behavior and neurobiological changes compared to the B6J strain based on previous studies (Bridgewater et al., 2017; Gelineau et al., 2017; Kuleshkaya et al., 2014; Yin et al., 2011). In this study, mRNA gene expression for brain-derived neurotrophic factor, dopamine receptor D2, and tyrosine hydroxylase was evaluated in the striatum, hippocampus, and olfactory bulb. Dopamine release and reuptake were assessed in the dorsal and ventral striatum. Behavior assessments included the open field test (for locomotion, velocity, habituation, and anxiety), nestlet shredding (for motivation, compulsivity, and welfare), and novel object recognition (for learning and memory).

CHAPTER II

LITERATURE REVIEW

Significance

The World Health Organization defines obesity as abnormal or excessive fat accumulation that may impair health. Approximately 13% of the global adult population was obese in 2016, and worldwide prevalence of obesity has nearly tripled since 1975 (“WHO | Obesity”). In the United States, obesity prevalence since 2016 was 39.8%, affecting 93 million adults (Hales et al., 2017). Biochemical consequences of obesity include inflammation, oxidative stress, and mitochondrial dysfunction, all of which are common pathologies of neurodegenerative disease (ND) (Mazon et al., 2017). Increasing evidence implicates obesity as a risk factor for two of the most common forms of ND: Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Martin-Jiménez et al., 2017; Mazon et al., 2017). The risk of developing a ND depends on a combination of genetic background and the environment (Brown et al., 2005). The mechanisms by which environmental factors, such as diet-induced obesity (DIO), can lead to ND are not fully understood. Trace element dysregulation and gene expression alterations have been associated with various NDs (Hwang et al., 2017; Mezzaroba et al., 2019) and with DIO (Han et al., 2019; Huang et al., 2005a; Lee et al., 2010), making it possible that these disease states share common mechanisms. Furthermore, obesity has been linked to

various behavioral disorders (Baker et al., 2017; Davidson et al., 2014) and dysregulated dopamine metabolism (Leite and Ribeiro, 2019). As conditions of overweight and obesity are increasing in both males and females worldwide, these alterations in behavior and biochemistry due to obesity can have serious consequences related to declining health, reduced productivity, and increased health care costs (Trogdon et al., 2008).

Trace Element Dysregulation in the Brain

Trace elements iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn) are essential for numerous physiological processes in humans and animals, such as energy production, synaptic transmission, and regulation of oxidative stress (Genoud et al., 2017; Peres et al., 2016). These metals participate in a variety of functions, serving as redox agents, enzyme cofactors, and stabilizers in protein structure (Genoud et al., 2017; Mezzaroba et al., 2019). The dysregulation of one or more of these trace elements can disrupt brain homeostasis and normal cellular processes, leading to neurodegeneration and disease (Mezzaroba et al., 2019). For example, Fe, Cu, and Zn disruptions in the hippocampus have been associated with AD (Cristóvão et al., 2016; Mezzaroba et al., 2019; Sensi et al., 2018). Fe accumulation and Cu depletion in the midbrain have been linked to PD (Belaidi and Bush, 2016; Liddell and White, 2018). Mn deficiency and Fe overload in the brain have been implicated in the development of Huntington's disease (HD) (Bryan and Bowman, 2017; P. Chen et al., 2019; Farina et al., 2013). In a study using post-mortem male and female human brain tissue, Fe

concentration was 25% higher in PD olfactory bulbs compared to controls (Gardner et al., 2017). Furthermore, in a study using brain tissue from AD patients, Fe and Zn were significantly elevated in the olfactory bulb (Samudralwar et al., 1995). Trace elements Fe, Cu, and Mn share some common transporters and enzymes, such as divalent metal transporter 1 and ceruloplasmin, allowing their uptake mechanisms to interact (Skjørringe et al., 2012; Ye et al., 2017). Therefore, a disruption in homeostasis of one element can impact the other elements, leading to potentially detrimental effects on the brain (Herrera et al., 2014; Skjørringe et al., 2012; Ye et al., 2017).

DIO Impact on Iron and Gene Expression in the Brain

Recently it has been discovered that DIO can lead to Fe dysregulation and gene expression disruption in Fe-rich brain regions (Han et al., 2019; Liu et al., 2016). In one study using male C57BL/6J (B6J) mice fed a high fat diet (HFD) for 20 weeks, DIO resulted in significant Fe reductions in the thalamus, increased Fe in the midbrain, and no effect on the hippocampus or striatum (Han et al., 2019). The expression of alpha synuclein increased by threefold in the midbrain, and heavy chain ferritin was reduced in the hippocampus to almost half the amount of the control. In another study using male B6J mice fed a HFD for 20 weeks, mice with DIO had significantly reduced Fe in the striatum, but no change in Fe in the hippocampus, midbrain, or thalamus (Liu et al., 2016). Total distance travelled, a measure of locomotion in rodents, was significantly reduced for DIO mice. Additionally, there was a positive correlation between midbrain Fe and sleeping time for mice fed a HFD. A survey of the current scientific literature

indicates that there is limited research regarding the effects of DIO on trace element status in the brain. The studies described here provide preliminary evidence for region-specific Fe dysregulation and gene expression alterations in the brains of male rodents fed a HFD, implicating DIO as a potential risk for neurodegeneration and behavior impediments.

Sex as a Biological Factor

The inclusion of sex as a biological factor in research studies is a key initiative proposed by the National Institutes of Health (Clayton, 2018). Supporting evidence for sex differences in response to a HFD can be found in several recent obesity studies. For example, in a study using male and female rats, males fed a HFD were found to be more prone to brain oxidative stress and trace element disruptions than females (Charradi et al., 2017). Specifically, there was a reduction in whole brain Mn and an increase in plasma Mn for male rats only. In a DIO study using male and female C57BL/6 (B6) mice, males fed a HFD displayed more anxiogenic behavior and had reduced locomotion compared to females (Bridgewater et al., 2017). Another study that examined neurodegeneration and brain connectivity in a group of patients with probable AD found that the effect of obesity on brain metabolism was more significant in females versus males (Malpetti et al., 2018). Based on research indicating sex differences in response to DIO, and in compliance with the NIH initiative, both males and females should be included in future DIO studies to evaluate sex as a biological variable.

Genetics as a Biological Factor

Genetic background is another important factor to consider in the study of DIO. Despite several studies that support a link between obesity and PD risk, some human qualitative studies reveal conflicting results. For example, obesity was associated with PD in both men and women in a Finnish cohort (Hu et al., 2006) and a South Korean cohort (Nam et al., 2018), yet no association was found in several large U.S. cohorts (Chen et al., 2004; Palacios et al., 2011). These discrepancies could be attributed to genetics when studying diverse populations. A DIO study using female B6J and DBA/2J (D2J) mice revealed distinct trends in gene expression in mice fed a HFD, with B6J mice upregulating glutathione peroxidase I and D2J mice downregulating glutathione reductase (Norris et al., 2016). Another study using different strains of male mice, including B6J and DBA/2 (D2) strains, found that while all mice became obese when fed a HFD, biomarkers for glucose homeostasis differed by strain (Montgomery et al., 2013). These human and animal studies highlight the unique responses to DIO based on genetics and imply a gene-environment interaction effect that should be further explored.

Combined Sex and Genetic Factors

The combined influence of sex and genetics on trace element dysregulation and gene expression has been described in aging and DIO research. For example, an aging study using male and female B6 and D2J mice found that aged D2J mice had greater whole-brain Fe levels compared to B6 mice, and sex differences were significant for D2J

mice only (Hahn et al., 2009). In a systems genetics report, recombinant inbred BXD strains derived from B6J and D2J progenitors were fed low Fe diets and evaluated for Fe and gene expression changes in the midbrain and striatum (Jellen et al., 2012). Fe was reduced in both brain regions but varied from 0-40% reduction depending on the strain. There was a diet by strain interaction in the midbrain, and a three-way interaction between diet, strain, and sex in the striatum. Differences in gene expression between males and females can impact susceptibility to disease in a sex-dependent manner (Torres-Rojas and Jones, 2018). For example, the *Sry* transcript, which regulates the enzyme tyrosine hydroxylase, is present in males but not females (Torres-Rojas and Jones, 2018). Environmental exposure to toxins that can downregulate the *Sry* gene will affect males only and could explain the increased risk for PD in males versus females. Collectively, these studies provide evidence that DIO or HFD could be considered a potential environmental stimulus for neurophysiological dysregulation, and may exert its effects in a sex and genetic-dependent manner. For this reason, the use of male and female subjects from different genetic backgrounds or strains should be considered for DIO investigations in the brain.

Behavior and DIO

Obesity has been linked to various behavioral and biochemical changes, such as reduced physical activity (Sanyaolu et al., 2019), increased anxiety (Baker et al., 2017), and compromised memory (Davidson et al., 2014). Furthermore, DIO can lead to gene expression alterations in the brain (Gan et al., 2015; Huang et al., 2005a; Wu et al.,

2017) and dysregulated dopamine metabolism (Leite and Ribeiro, 2019). A brief review of the current literature describing the relationship between obesity and each of these categories is provided below.

DIO and Physical Activity

Physical activity or mobility can be impacted by a state of obesity in humans and rodents. In humans, obesity has been associated with decreased fine motor control and speed (C. Wang et al., 2016), reduced functional mobility in adults (Forhan and Gill, 2013; Trivedi et al., 2015), and decreased physical activity in children and adolescents (Sanyaolu et al., 2019). In rodents, there are mixed results. In some studies, B6J and B6 mice fed a HFD show reduced locomotion due to HFD (Almeida-Suhett et al., 2017; Krishna et al., 2016; Tsai et al., 2018; Wu et al., 2018), while other studies show no impact of diet on physical activity (Bridgewater et al., 2017; Zilkha et al., 2017). Furthermore, there are known sex effects that impact mobility in humans and rodents (Rosenfeld, 2017). In children and adolescents, boys generally show higher physical activity levels compared to girls (Rosenfeld, 2017). On the contrary, female rodents tend to have higher activity levels compared to males. Sex differences in physical activity or locomotion in the context of obesity have also been reported. In a cross-sectional study in 964 community dwelling older adults, obese women were found to be less active than obese men (Gretebeck et al., 2017). In B6J and B6 mice, male mice fed a HFD are frequently reported as having reduced locomotion in an open field (Almeida-Suhett et al., 2017; Gelineau et al., 2017; Tsai et al., 2018; Wu et al., 2018), while female

mice show mixed results, with some having decreased locomotion (Krishna et al., 2016), some increased locomotion (Krishna et al., 2015), and others with no effect (Bridgewater et al., 2017; Gelineau et al., 2017). These discrepancies may be due to the duration of diet treatment, age of behavioral testing, and diet composition.

The striatum is a primary regulator of spontaneous physical activity (Rosenfeld, 2017). Although the precise mechanisms of how DIO can impact mobility are not clear, it is possible that DIO may disrupt the expression of genes such as dopamine receptor D2 and tyrosine hydroxylase, which are associated with dopamine and physical activity (Gallo, 2019; Jang et al., 2017). Furthermore, it is possible that DIO may disrupt dopamine signaling in the striatum. A discussion of dopamine and dopamine-related genes is discussed in more detail later in this chapter.

DIO and Anxiety

Obesity has been associated with a higher prevalence of anxiety, as demonstrated in several human studies (Baker et al., 2017; Garipey et al., 2010; Strine et al., 2008) and rodent studies (Almeida-Suhett et al., 2017; Krishna et al., 2016). For example, a cross-sectional study of 217,379 adults in the United States found a positive association between obesity and anxiety (Strine et al., 2008). Similarly, a study in the United States with 9125 adults found that obesity lead to an approximate 25% increase in odds of having an anxiety disorder (Simon et al., 2006). In other populations, a meta-analysis in China that included 17,894 children and adolescents found a significantly higher incidence of anxiety in obese and overweight subjects (40%) compared to normal

weight subjects (14%) (Wang et al., 2019). Furthermore, anxiety was also found to be associated with obesity during pregnancy and the postpartum stage, increasing health risks for mothers who have gained excessive weight beyond normal pregnancy weight (Nagl et al., 2015). In rodent DIO studies, B6J male mice (Almeida-Suhett et al., 2017) and B6 female mice (Krishna et al., 2016) fed a HFD displayed higher anxiety-like behavior in an open field as assessed by decreased center time. Additionally, male Fischer 344 rats (Buchenauer et al., 2009) and female Long Evans rats (Sivanathan et al., 2015) fed a HFD also exhibited more anxiety-like behavior compared to normal weight rats fed a control diet. In contrast, there are other reports in humans and rodents that found no link between obesity and anxiety (Araujo et al., 2017; Gelineau et al., 2017; Tsai et al., 2018). The relationship between obesity and anxiety is complex, often due to comorbidities and a potential bidirectional association (Baker et al., 2017). More research is needed to understand the physiological mechanisms that may connect obesity to anxiety.

While there is extensive research on sex differences regarding associations between obesity and depression, less is known about sex factors involved in relationships between obesity and anxiety (Tronieri et al., 2017). Some studies show a more significant relationship between overweight or obesity and anxiety in females compared to males (Anderson et al., 2006; Barry et al., 2008; DeJesus et al., 2016; Hofmann et al., 2015; Svenningsson et al., 2012). However, there are other reports that suggest a higher incidence of anxiety in obese males compared to obese females

(Bjerkeset et al., 2007; Bridgewater et al., 2017; Tronieri et al., 2017). More work is needed to understand the physiological mechanisms involved and the influence of sex on this relationship between obesity and anxiety.

The relationship between obesity and anxiety may also depend on genetics. A cross-sectional study in the Midwest found a positive association between BMI and anxiety in Caucasians and African Americans, but not in Asians or Hispanics (DeJesus et al., 2016). An epidemiological study in the United States found that the correlation between obesity and a specific form of anxiety depended on ethnicity (Rosen-Reynoso et al., 2011). For example, obesity was associated with African Americans with general anxiety disorder, non-Latino whites with panic disorder, Latinos with agoraphobia without panic disorder, and Asians with post-traumatic stress disorder. In contrast, one study found no significant difference across ethnic groups when comparing the association of obesity and anxiety in Caucasians, African Americans, and Latinos (Bodenlos et al., 2011). Compared to literature that describes the influence of sex on the relationship between obesity and anxiety, there is less information available regarding the impact of ethnicity or genetics on this relationship. More human studies on this topic should stratify by ethnicity, and more rodent studies should include strain comparisons to address this gap in the literature.

DIO and Memory

Obesity may also have a negative impact on memory. In a study of 513 Malaysian adolescents, high body mass index (BMI) was associated with poor working

memory (Tee et al., 2018). A study in 60 adults <41 years old found that BMI was negatively associated with working memory, but not with learning and memory (Coppin et al., 2014). In a secondary analysis of older adults (average age of 74) comprising 701 normal weight, 1,082 overweight, and 902 obese individuals, the obese group had a statistically significant lower memory training score compared to the normal weight group (Clark et al., 2016). An extensive review of the impact of HFD on learning and memory in rodent studies using various test measures found that most studies, but not all, found an association between HFD or DIO and memory decline (Cordner and Tamashiro, 2015). Taken together, most of these studies show a pattern of obesity with reduced memory, but few distinguish between males and females or differences in genetics that may cause discrepancies in the results.

DIO and Dopamine

Dopamine plays an important role in regulating motor control, cognition, and motivation (Mishra et al., 2018). Previous DIO studies in mice have found an inverse relationship between dopamine signaling with body weight (Zilkha et al., 2017) and DIO-induced anxiety with dopamine turnover in the brain (Krishna et al., 2015). In both the dorsal and ventral striatum, the regulation of dopamine neurotransmission is implicated as a modulator of DIO and food reward (Baik, 2013). For example, in a study with male B6J mice fed a high fat/high sugar Western style diet for 16 weeks, dopamine release in the dorsal striatum was increased and dopamine clearance in the dorsal striatum was decreased in mice fed the Western style diet (Fritz et al., 2018). In the nucleus

accumbens (NAc) core of the ventral striatum, dopamine release assessed by fast scan cyclic voltammetry was increased in male Sprague-Dawley rats when exposed to a sucrose-based food reward (Roitman, 2004). In a study with male B6J mice fed a HFD for six weeks, voltammetry measurements revealed a significant decrease in dopamine reuptake in the NAc core (Fordahl and Jones, 2017). The NAc core of the ventral striatum is involved in the mediation of reward, satisfaction, and motivation, and has been implicated in numerous behavioral disorders, such as anxiety, obsessive-compulsive disorder, and addiction (Salgado and Kaplitt, 2015). The dorsal striatum is involved in habitual and compulsive behaviors such as food-seeking and binge eating, and plays a role in homeostatic energy consumption (Fritz et al., 2018). Both regions are important to consider when investigating dopamine metabolism as it relates to DIO.

Sex Factors in Murine Behavior

Research that includes both sexes in the study of DIO-impact on behavior in mice is limited. In one study using male and female B6 mice fed a HFD (60% kcal fat) for 12 weeks, males fed a HFD spent less time in the center zone of the open field compared to males fed a control diet and compared to all females, indicating increased anxiety-like behavior in males only due to HFD (Bridgewater et al., 2017). Additionally, there was a sex difference in ambulation, with male B6 mice showing reduced locomotor activity compared to B6 females. Interestingly, diet had no effect on locomotion for either sex. In contrast to these results, a study using male and female B6J mice fed a HFD (60% kcal from fat) for approximately 10 weeks found no significant difference in open field center

time due to diet or sex (Gelineau et al., 2017). Male B6J mice fed a HFD had reduced locomotion compared to the male LFD group, yet there was no diet impact on female locomotion, and no overall sex differences in locomotion. There was, however, a sex difference in protein expression of brain-derived neurotrophic factor (BDNF), with males exhibiting lower expression compared to females. There was no significant impact of diet on BDNF expression for either sex. While both studies report an impact of diet or sex on various behaviors, the results are not consistent. Both studies initiated the HDF at a similar age (6-7 weeks), but the diet treatment duration was different (12 weeks versus 10 weeks). The anxiety-like behavior in males was revealed after the 12-week diet treatment, which could be a result of increased body weight, older age, or extended exposure to the HFD.

Gene Expression Dysregulation in the Brain

DIO has been associated with gene expression dysregulation in the brain, with implications for the development of ND or behavior impairments (Han et al., 2019; Huang et al., 2005; Lee et al., 2010). There is also potential for a bidirectional relationship between gene expression alterations and trace element dysregulation. The genes or proteins described here are relevant for the investigation of the impact of DIO on the brain, together with the sex and genetic factors that may influence the outcome.

Divalent Metal Transporter 1

Divalent metal transporter 1 (DMT1) acts as a transporter for several ions, including ferrous ion (Fe^{2+}), manganese II (Mn^{2+}), cobalt II (Co^{2+}), cadmium II (Cd^{2+}),

nickel II (Ni^{2+}), lead II (Pb^{2+}), and to a lesser extent zinc (Zn^{2+}) (Mackenzie et al., 2007).

Both cuprous (Cu^+) and cupric ion (Cu^{2+}) are transported by DMT1, although Cu^+ has a higher affinity and can compete with Fe^{2+} (Arredondo et al., 2003). There are four known isoforms of DMT1 with similar function, all of which transport Fe^{2+} with similar efficiency (Skjørringe et al., 2015). DMT1 is mainly expressed in neurons, but the level of expression and compartmentalization of DMT1 within non-neuronal cells, such as astrocytes, is still controversial (Ingrassia et al., 2019; Skjørringe et al., 2015).

Investigations are needed to learn more about the gene expression of DMT1 in the brain under DIO conditions, as the dysregulation of DMT1 and trace element homeostasis is associated with various NDs (Ingrassia et al., 2019; C.-W. Zhang et al., 2017).

Several studies in rodents have shown an association between dysregulated DMT1 expression and alterations in Fe or Cu homeostasis in a brain region-specific manner. A study using male Sprague-Dawley rats found that Fe-related protein expression changes with age at different rates depending on the brain area (Lu et al., 2017). In the striatum, substantia nigra, hippocampus, and cortex, the Fe importer DMT1 was upregulated with age. While the increase in DMT1 in the striatum and hippocampus occurred mainly between three-12 months of age, the increase in the substantia nigra and cortex occurred between 12-24 months. The accumulation of Fe in each region occurred over the entire time period (three–24 months), suggesting that DMT1 upregulation may influence Fe build up to some degree, but is not the only factor involved in Fe accumulation. Unlike DMT1, transferrin receptor 1 (TfR1) was

downregulated with age. These differences suggest that Fe accumulation over time is more likely to depend on the expression of DMT1 versus TfR1. In a study using male *+/b* (control) and *b/b* (DMT1 mutated) Belgrade rats, DMT1-mutated rats displayed a reduction of Fe in the striatum, hippocampus, olfactory bulb, and cortex, as well as an increase in Cu in the striatum and hippocampus (Han et al., 2016). It was found that transgenic mice overexpressing DMT1 in multiple brain regions (striatum, substantia nigra, hippocampus, olfactory bulb, and cortex) accumulated Fe in the substantia nigra when fed an Fe-supplemented diet (C.-W. Zhang et al., 2017). Interestingly, there was no corresponding increase in alpha synuclein, and motor function was similar between the treatment group and control mice. The results of this study show that an increase in Fe is only one factor that may contribute to the development of neurodegeneration, increasing the challenge of elucidating the pathological mechanisms of ND.

The effect of DIO on DMT1 expression in the context of Fe metabolism has been studied systemically, yet there is limited research focused on the brain. A recent study showed that DIO did not induce DMT1 gene expression changes in various brain regions in male B6J mice (Han et al., 2019). In the system, it was found that male B6 mice fed a HFD (60% kcal fat) for eight weeks showed reduced plasma Fe and increased DMT1 mRNA and protein expression in the duodenum compared to control mice, indicating a systemic Fe deficiency caused by DIO (Sonnweber et al., 2012). In contrast, another study found that B6J male mice fed a HFD (54% kcal fat) for 30 weeks resulted in no change in duodenal DMT1 mRNA or protein expression, but had Fe accumulation in the

liver and spleen (Citelli et al., 2015). In male Swiss mice fed a HFD (60% kcal fat), DMT1 expression in adipose tissue was not affected (Gotardo et al., 2013). Collectively, these results show that gene expression alterations and Fe dysregulation due to DIO may be tissue-specific, and may depend on the length of time exposed to HFD. The effects of DIO on DMT1 gene expression reported in the literature have focused primarily on male rodents and the system, leaving a knowledge gap on DIO impact in the brain and the effect of sex on potential gene expression alterations.

Iron Regulatory Protein

Iron regulatory protein (IRP) is integral in the regulation Fe absorption, transportation, and storage at the cellular level (Zhou and Tan, 2017a). The two isoforms, IRP1 and IRP2, are both involved in Fe homeostasis. When intracellular Fe levels are low, IRP binds to an iron response element (IRE) on the mRNA transcripts of Fe-metabolism proteins, such ferritin, ferroportin, transferrin receptor1, and DMT1. The binding of IRP inhibits the translation of ferritin and ferroportin, and protects TfR1 and DMT1 from degradation. When Fe levels are high, Fe binds to IRP, causing the release of IRP from the IRE. This leads to the synthesis of ferritin and ferroportin to stimulate Fe storage or cellular efflux, and promotes the degradation of TfR1 and DMT1 mRNA transcripts. Disruptions in gene expression of IRP1 or IRP2 can therefore impact Fe homeostasis, as these regulatory proteins control the synthesis of several Fe-related proteins.

It has been discovered that there is an IRE present in alpha synuclein mRNA transcripts (Cahill et al., 2009), and one in amyloid precursor protein mRNA transcripts that binds specifically with IRP1 (Bandyopadhyay and Rogers, 2014; Cho et al., 2010). IRP binding to either of these IREs suppresses protein translation (Cahill et al., 2009; Zhou and Tan, 2017a). Fe overload can therefore lead to the translational upregulation of amyloid precursor protein (Cho et al., 2010) or alpha synuclein (Febbraro et al., 2012), increasing the risk for ND (Cahill et al., 2009). An effect of sex for this relationship between IRP and alpha synuclein was found in rats, as females expressed more IRP1 in the hippocampus compared to males, and had reduced expression of alpha synuclein (Thulluri et al., 2012). There is limited research on the effects of DIO on brain IRP expression. In the system, it was found that IRP1 (but not IRP2) mRNA expression was upregulated in adipose tissue of male Swiss mice fed a HFD (60% kcal fat) (Gotardo et al., 2013). Research on the effect of DIO on the gene expression of IRP1 should be pursued, as this protein is instrumental in Fe homeostasis through its impact on several Fe-related proteins. Furthermore, the relationship between IRP1 and alpha synuclein or amyloid precursor protein may have important implications for the development of ND, and should be studied in the context of DIO.

Ceruloplasmin

Ceruloplasmin is a Cu-dependent protein that is expressed in astrocytes at the blood brain barrier and in dopaminergic neurons in the substantia nigra (Hellman and Gitlin, 2002). It acts as a ferroxidase, allowing for the efflux of Fe from astrocytic cells to

neurons via transferrin. Cu depletion can therefore lead to Fe dyshomeostasis, trapping the Fe within astrocytes, and depriving neurons of Fe. This enzyme also acts as an oxidizing agent for Mn, and can function as a general endogenous antioxidant (Hellman and Gitlin, 2002; Jursa and Smith, 2009). Ceruloplasmin is critical for the maintenance of Fe homeostasis, and can oxidize 6-hydroxydopamine without producing reactive oxygen species as an end product (Vassiliev et al., 2005). A deficiency in ceruloplasmin can lead to dysregulated Fe and production of reactive oxygen species (ROS) by either the Fenton reaction of Fe^{2+} with hydrogen peroxide to produce the deleterious hydroxyl radical, or by the nonenzymatic oxidation of 6-hydroxydopamine to produce hydrogen peroxide. Consequently, aberrations in ceruloplasmin gene expression or protein synthesis have been associated with ND. Murine ceruloplasmin is 90% similar to human ceruloplasmin and has similar gene expression patterns, allowing for the use of a mouse model to study human ND (Hellman and Gitlin, 2002).

Ceruloplasmin levels tend to be higher in the plasma of obese individuals (Kim et al., 2011; Yang et al., 2019). Although there is limited research on the impact of DIO on ceruloplasmin expression in the brain, there is extensive research using rodent and human models to investigate the role of ceruloplasmin in ND. For example, a study using male Sprague-Dawley rats found that ceruloplasmin mRNA expression increased with age, with a more extensive induction in the midbrain and striatum compared to the hippocampus and cortex (Chang et al., 2005). In male Wistar rats, ischemia led to decreased mRNA expression of ceruloplasmin in the hippocampus, with corresponding

accumulation of Fe in neurons (Li et al., 2008). In mice, ceruloplasmin mRNA and protein was upregulated in the retinas of glaucomatous D2 mice, but not B6 control mice (Stasi et al., 2007). A study using postmortem brain tissue from ND patients found increased levels of ceruloplasmin protein in AD striatum, HD midbrain, and the hippocampus for AD, PD, and HD tissue compared to elderly controls (Loeffler et al., 1996). Moreover, it was discovered that ceruloplasmin was depleted in the hippocampus of AD postmortem tissue and in an AD mouse model, and that restoration of ceruloplasmin alleviated neuronal damage (Zhao et al., 2018). Collectively, these studies show that dysregulated ceruloplasmin expression is associated with the aging process and various forms of neurodegeneration. It is possible that DIO may also disrupt the normal expression of ceruloplasmin in the brain.

Ceruloplasmin dysregulation may also be related to behavior changes. In ceruloplasmin knockout mice, Fe was reduced in the hippocampus and mice exhibited higher anxiety-like behavior in an open field test (Texel et al., 2012). These mice also had a reduction in BDNF expression in the hippocampus. In male and female patients with obsessive compulsive disorder (OCD), serum ceruloplasmin levels were found to be elevated, suggesting a potential association between ceruloplasmin homeostasis and OCD (Virit et al., 2008). In a study with patients diagnosed with aceruloplasminemia, a serious ND involving the lack of synthesis of ceruloplasmin, nearly half of the patients presented with anxiety and depression (Vroegindeweij et al., 2017). While none of these studies definitively tie ceruloplasmin to a specific behavior, the evidence suggests

that ceruloplasmin dysregulation and concomitant Fe accumulations may be associated with various psychotic disorders, warranting more research to be performed.

Copper Transporter 1

Copper transporter 1 (CTR1) is a plasma membrane protein expressed in the blood brain barrier (BBB), the blood cerebrospinal fluid barrier (BCB), neurons, and astrocytes (Skjørringe et al., 2012). Through CTR1, Cu import occurs mainly at the BBB, and Cu export mainly at the BCB (Montes et al., 2014). Previously, it has been shown that CTR1 expression correlates with increased Cu level in the substantia nigra in PD. Since Cu dysregulation has been implicated in the development of various NDs (Genoud et al., 2017; Mezzaroba et al., 2019; Sensi et al., 2018), the effect of DIO on gene expression of CTR1 and its potential link to DIO-induced neurodegeneration should be investigated.

Although there is limited information available regarding the impact of DIO on CTR1 expression, studies using various models have shown connections between the dysregulation of CTR1 expression and Cu homeostasis with neurodegeneration. In postmortem substantia nigra brain tissue from PD and AD patients, there were significant reductions in Cu, CTR1 protein, and superoxide dismutase 1 (SOD1) (Davies et al., 2014). The reductions in SOD1 were positively correlated with Cu reductions, and Cu decline occurred before neuronal death. This study provides evidence that the dysregulation of Cu and CTR1 are associated with oxidative stress and neurodegenerative damage. Lang et al. used a *Drosophila* AD model to evaluate the

impact of Cu and CTR1 gene expression on neurodegeneration (Lang et al., 2013).

Downregulation of CTR1 resulted in reduced levels of Cu, but not Fe, Mn, and Zn. Flies with higher Cu levels experienced more beta amyloid toxicity as assessed by locomotion and mortality. CTR1 gene expression and Cu concentration did not, however, have an impact on memory. Zheng et al. found that mouse BV-2 microglial cells treated with IFN- γ , a proinflammatory cytokine, resulted in Cu accumulation and induction of CTR1 mRNA gene expression (Zheng et al., 2010). The implications of this study are significant to the health field, as inflammation is associated with ND (Heneka et al., 2015; Ransohoff, 2016) and with DIO (Saltiel and Olefsky, 2017). CTR1 and Cu homeostasis can also be influenced by Mn exposure. In a study using the Z310 choroidalepithelial cell line from murine choroid plexus, exposure to Mn resulted in increased cellular Cu and upregulated CTR1 and DMT1 gene and protein expression (Zheng et al., 2010). This study highlights the interplay between trace elements and their effect on gene expression and homeostasis. Collectively, these studies provide evidence for the potential impact of CTR1 expression and Cu regulation in the development of ND. The potential impact of DIO on CTR expression in the brain has not thus far been investigated.

Alpha Synuclein

Alpha synuclein, encoded by the *SNCA* gene (Fitzgerald et al., 2019), is expressed throughout several brain regions and is enriched within presynaptic terminals (Bridi and Hirth, 2018). It acts as a negative modulator of dopamine by inhibiting enzymes that

synthesize dopamine, such as tyrosine hydroxylase. The overexpression of alpha synuclein mRNA transcripts may lead to the aggregation of alpha synuclein protein (Fields et al., 2019), and can disrupt the function of the presynaptic SNARE complex, interfering with the positioning and fusion of synaptic vesicles (Bridi and Hirth, 2018). Additionally, accumulated alpha synuclein in the form of Lewy bodies can lead to the dysregulation of dopamine, synaptic dysfunction, and damaged neurons, provoking the development of neurodegenerative diseases such as PD (Bridi and Hirth, 2018; Fields et al., 2019; Fitzgerald et al., 2019).

There are several biochemical interactions between alpha synuclein and Fe in the brain (B. Chen et al., 2019; Lingor et al., 2017). Fe can regulate alpha synuclein levels post transcriptionally through the binding of IRP on the alpha synuclein IRE, and post translationally by interfering with the normal ubiquitination process of alpha synuclein protein (B. Chen et al., 2019). It has been proposed that alpha synuclein can also regulate Fe levels through its ferredoxinase activity, a process that depends on Cu as a cofactor. Over expression of alpha synuclein in both cell and animal models increases the intracellular reduction of ferric ion to ferrous ion, thereby increasing the risk for ROS generation through the Fenton reaction. Furthermore, the overexpression of alpha synuclein in midbrain neurons was reported to increase intracellular Fe (Ortega et al., 2016) and intracellular Mn (Dučić et al., 2015), but had no effect on Zn concentrations (Dučić et al., 2015).

Recent studies in rodents and cells demonstrate the impact of DIO on alpha synuclein expression and neuropathology. For example, male B6J mice fed a HFD (60% kcal fat) for 20 weeks showed increased alpha synuclein mRNA and protein expression in the midbrain compared to LFD control mice (Han et al., 2019). Additionally, Fe and F2-isoprostane (a biomarker for oxidative damage) levels were also elevated in this brain region. A study using female C57BL/6J/129SVJ mice fed a HFD (58% kcal fat) for 12 weeks found an upregulation of alpha synuclein mRNA in the hypothalamus (Lee et al., 2010). In a study using male ApoE^{-/-} and ApoE^{-/-}/Tollip^{-/-} mice, HFD (42% kcal fat) led to the accumulation of alpha synuclein and beta amyloid protein in the hippocampus and increased neuronal death (Chen et al., 2017). Together, these studies provide evidence that both male and female mice are prone to dysregulated alpha synuclein expression due to DIO. Additionally, a HFD may instigate alpha-synucleinopathy in mice that are genetically predisposed to age-dependent alpha synuclein pathology. For example, a study using male B6 transgenic mice expressing human mutant [A30P] alpha synuclein fed a HFD (45% kcal fat) demonstrated that HFD-induced obesity accelerates alpha-synucleinopathy and astrogliosis (Rotermund et al., 2014). Cell studies also show a potential relationship between alpha synuclein dysregulation and HFD. In a study using SH-SY5Y human neuroblastoma cells as a neuronal model, and T98G human glioblastoma cells as an astrocytic model, alpha synuclein unexpectedly reduced the cytotoxic effects of palmitic acid, a long chain saturated fatty acid (Ng and Say, 2018). In contrast, a study using U373 MG human astrocytoma cells found that overexpression

of alpha synuclein triggered oxidative stress and cell death in these glial cells (Stefanova et al., 2001). While these cell studies show direct effects of saturated fat or alpha synuclein overexpression on brain cell viability, further studies are required to understand the effect of HFD-feeding on the mRNA expression of alpha synuclein and its potential connection to trace element dysregulation and neurodegeneration.

The dysregulation of alpha synuclein, particularly in the hippocampus, has been implicated in the disruption of normal behavior. Alpha synuclein pathology in the hippocampus was associated with memory loss in patients with Dementia with Lewy Bodies (Adamowicz et al., 2017). In rodents, it was found that when comparing Lewis rats to spontaneously hypertensive rats, the Lewis rats expressed higher levels of anxiety in an open field test and had higher concentrations of alpha synuclein and beta amyloid in the hippocampus (Chiavegatto et al., 2009). Moreover, alpha synuclein was inversely correlated to dopamine turnover. This study suggests that alpha synuclein may be involved in regulating anxiety-like behaviors in rodents through dopaminergic mechanisms.

PD is characterized by intracellular aggregates of alpha synuclein in the form of Lewy bodies, localized in the substantia nigra (Ubeda-Bañon et al., 2013). It has been discovered recently that alpha synuclein accumulates in the olfactory bulb long before it does in the substantia nigra, and is associated with the loss of olfactory sense in PD (Adler and Beach, 2016; Fullard et al., 2017) and AD (Attems et al., 2014). Analysis of postmortem brain tissue revealed that alpha synuclein was accumulated in male and

female olfactory bulbs in patients with PD patients and confirmed Lewy body formation (Braak et al., 2003; Mazurskyy and Howitt, 2019). Additionally, the olfactory bulb was determined in autopsy studies to be the first brain region affected by Lewy type alpha synucleinopathy (Adler and Beach, 2016). The effect of DIO on alpha synuclein expression or trace element dysregulation in the olfactory bulb is unknown, but could potentially provide valuable information regarding the development of ND in the context of obesity.

Amyloid Precursor Protein

Amyloid precursor protein (APP) is a transmembrane protein associated with several biological functions, including cellular proliferation and differentiation, cell-fate specification, and neurite outgrowth (S. Wang et al., 2016). The overexpression of APP, however, has been implicated in the progression of AD (Roher et al., 2017). Common features of AD include extracellular accumulation of senile plaques, intracellular neurofibrillary tangles, and loss of neurons and synapses in the brain (Zhang et al., 2011; Zheng and Koo, 2011). The senile plaques consist mainly of beta amyloid protein, which is derived from the proteolytic cleavage of APP. Disruptions in normal gene expression of APP can therefore lead to the potential buildup of beta amyloid, promoting the conditions for neurodegeneration.

APP is highly expressed in both the hippocampus and olfactory bulb in rodents (S. Wang et al., 2016). The hippocampus is particularly vulnerable to beta amyloid protein aggregation and senile plaque formation (Zhang et al., 2011). While there are

several studies examining the effects of aging on APP gene expression and beta amyloid production in the brain, only a few studies have focused on the effects of diet and obesity. In one report using male B6J mice fed a HFD based on palmitic acid for 16 months, beta amyloid protein was found to accumulate in the hippocampus (Busquets et al., 2017). The authors suggest that the beta amyloid accretion may be due to impeded autophagy of this protein. In another study using male B6J mice fed a HFD based on milk fat for 22 weeks, mice fed the HFD had elevated APP expression in the hippocampus (Puig et al., 2012). Beta amyloid aggregation in the olfactory bulb is also implicated in the progression of neurodegenerative disorders based on post mortem studies (Attems et al., 2014). In a study using Tg2576 AD mice, APP gene overexpression in the olfactory bulb impaired the function of protein kinase A between 6-18 months (Lachen-Montes et al., 2019). The authors speculate that this dysregulation of biochemical activities in the olfactory bulb supports the early progression of AD. There is limited data for the expression of APP in the human olfactory bulb (Rey et al., 2018), and no reports on the effect of obesity or diet on APP expression in the olfactory bulb.

There are conflicting reports concerning the relationship between Cu concentration in the brain with APP gene expression. In mouse neuroblastoma N2a cells, Cu treatments promoted an increase in APP mRNA expression and beta amyloid synthesis in a dose-dependent manner (Hou et al., 2015). Cu overload was associated with APP upregulation in fibroblast cells from B6 mutants (Armendariz et al., 2004), and depleted Cu was correlated to reduced APP expression in human fibroblast cells with

MNK deletion (Bellingham et al., 2004). In contrast, a study using SH-SY5Y neuronal cells found that a Cu surplus causes a delocalization of APP by increased exocytosis and reduced endocytosis, but not via APP gene expression (Acevedo et al., 2011).

Additionally, this study showed that Fe and Zn did not impact APP homeostasis. Taken together, these studies provide evidence that APP and beta amyloid homeostasis can be disrupted by different mechanisms, warranting more research to understand the neuropathology behind DIO and APP dysregulation.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a protein and growth factor involved in neuronal survival and brain plasticity (Bathina and Das, 2015; Miranda et al., 2019).

The role of this neurotrophin in brain plasticity is correlated with learning, memory, and cognition in humans and rodents (Miranda et al., 2019). Protein and mRNA expression of BDNF has been identified in brain regions such as the hippocampus, cortex, and olfactory bulb (Bathina and Das, 2015). High levels of BDNF are associated with neuronal protection (Almeida et al., 2005), while low levels have been associated with normal aging and pathological conditions such as AD, PD, and HD (Bathina and Das, 2015; Miranda et al., 2019). In a study using postmortem brain tissue from AD patients, BDNF mRNA expression was downregulated in the hippocampus and cortex compared to age-matched controls (Hock et al., 2000). Furthermore, BDNF is involved in the regulation of energy balance, and may act as an anorexigenic signaling molecule (Liu et

al., 2014; Rios et al., n.d.). As such, decreased levels of BDNF have been associated with obesity (Genzer et al., 2016).

Several reports using male rodents indicate that BDNF expression can be influenced by a HFD. One study using B6 male mice fed two types of HFD (41% kcal fat for 21 weeks or 60% kcal fat for 16 weeks) discovered that while BDNF expression and cognition were not impacted with the 41% HFD treatment, mice fed the 60% HFD had reduced levels of BDNF protein in the cortex and poor cognition performance compared to mice fed a control diet (Pistell et al., 2010). Another study using B6 males fed a HFD (42% kcal fat) for 12 weeks found decreased BDNF expression in whole brain tissue, but found no change in cognition or memory (Wang et al., 2017). In contrast, some rodent studies show an upregulation of BDNF due to HFD. For example, a study using male B6 mice fed a HFD (42% kcal fat) for eight weeks found that BDNF mRNA and protein was upregulated in whole brain tissue and in HT-4 hippocampal neurons (Genzer et al., 2016). In a study using male Long-Evans rats fed a HFD (45% kcal fat) for a short time frame of 72 hours, the mRNA expression of BDNF was upregulated in the hippocampus (Gan et al., 2015). Although there are discrepancies revealed here showing either induction or repression of BDNF, overall there is a consistent dysregulation in BDNF expression when rodents are fed a HFD. Likewise, behavior tests show that cognition is not always impacted by HFD and may depend on the age of the rodents, duration of the HFD, and type of assessment used.

Fewer investigations have been performed with female rodents. One study using female Fisher 344 rats fed a diet high in fat (39% kcal fat) and sugar evaluated BDNF expression in the hippocampus and caudal cerebral cortex after two months, six months, and two years of diet treatment (Molteni et al., 2002). At all three time points, mRNA expression was reduced in the hippocampus for rats fed a high fat and sugar diet compared to the control diet. Protein was also repressed in the hippocampus after six months of diet treatment. There was no impact of diet on mRNA or protein expression in the cortex. Additionally, rats fed the high fat and sugar diet had poor performance in spatial learning tasks.

There are notable sex differences in the distribution of BDNF within different brain structures (Chan and Ye, 2017). In rats, females have higher levels of BDNF in the hippocampus and cortex. In humans, there is no difference in BDNF levels in the hippocampus, but females have higher levels of BDNF in the prefrontal cortex compared to males. A study using Long-Evans male and female rats fed a control diet or HFD (45% kcal fat) for four days and four weeks compared BDNF mRNA expression in the ventromedial nucleus of the hypothalamus (Liu et al., 2014). At both time points, the expression of BDNF was higher in females compared to males, regardless of diet. After four days of HFD-feeding, there was no impact on BDNF expression. However, after four weeks, BDNF mRNA was repressed only in male rats fed a HFD.

There are very few studies that address the influence of strain and sex on DIO-induced BDNF gene expression dysregulation, and no reports describing the effects of

HFD on BDNF expression in the olfactory bulb. An evaluation of HFD impact on BDNF mRNA expression in the hippocampus and olfactory bulb in male and female mice of different strains would address these gaps in the literature.

Tyrosine Hydroxylase

Tyrosine hydroxylase (TH) is a rate limiting enzyme that requires Fe as a cofactor for the synthesis of catecholamines such as dopamine, epinephrine, and norepinephrine (Daubner et al., 2011). These catecholamines serve brain functions such as attention, memory, and cognition. TH is present within the neurons of the dopaminergic pathway that extends from the substantia nigra to the striatum, which is imperative for proper motor function (Jang et al., 2017). Obesity has been shown to have a negative impact on mobility (Forhan and Gill, 2013) and has been associated with changes in TH gene expression in different brain regions (Huang et al., 2005a; Lee et al., 2010; Ong et al., 2013; Wu et al., 2017).

Several studies have used animal models to investigate the impact of DIO on TH mRNA and protein expression. In a study using male B6 mice fed a HFD (40% kcal fat) for 20 weeks, there was an upregulation of TH mRNA in the midbrain for mice fed a HFD, and a positive correlation between final body weight and TH gene expression in this brain region (Huang et al., 2005a). In contrast, a study that also used B6 males fed a HFD (40% kcal fat) for 14 weeks found that mice fed a HFD had downregulated TH in the midbrain (Li et al., 2009). Both studies originated from the same lab and both began the diet treatment at 12 weeks of age, but the duration of HFD feeding differed (20 weeks

versus 14 weeks), as well as the age of mice at the time of tissue analysis. Another study using male B6 mice fed a HFD (60% kcal fat) for 13 weeks found that TH protein expression was downregulated in the midbrain and striatum for mice fed a HFD (Jang et al., 2017). Additionally, these mice had 60% reduced movement in the open field test. Corroborating these results, B6J male mice fed a HDF (58% kcal fat) for just 6 weeks also showed a decrease in TH protein expression in the striatum and midbrain, along with increased anxiety-like behavior in mice fed a HFD (Sharma et al., 2013). A study using female C57BL6/129SVJ mice fed a HFD (58% kcal fat) for 12 weeks discovered an induction of TH mRNA expression in the hypothalamus using microarray analysis and real time polymerase chain reaction techniques (Lee et al., 2010).

TH gene expression dysregulation has also been examined in humans with overweight and obesity. In a study using postmortem brain tissue from male and female Caucasians and African Americans, TH mRNA was downregulated in the substantia nigra in obese tissue, but not overweight or control tissue (Wu et al., 2017). Additionally, TH mRNA was negatively correlated with BMI, but not with age, sex, or race. In this same study, there was no significant change in dopamine receptor D2 mRNA expression.

Sex differences have been reported for the dysregulation of TH gene expression due to HFD. In male and female Albino Wistar rats fed a cafeteria style HFD (43% kcal fat), there was a diet by sex interaction for the expression of TH mRNA in the NAc core (Ong et al., 2013). TH was downregulated in males but upregulated in females.

Additionally, there was no significant difference in dopamine receptor D2 expression for males, but a repression in females was approaching statistical significance.

While several reports provide information about DIO-induced dysregulation of TH mRNA expression in the midbrain or TH protein expression in the striatum and midbrain, there is very little information regarding TH mRNA expression in the striatum, or other brain regions such as the olfactory bulb in the context of HFD. Furthermore, most studies with rodents are focused on males, and sex and strain differences are generally not addressed.

Dopamine Receptor D2

There are five major dopamine receptor subtypes, D1-D5, which belong to either the D1-like or D2-like receptor family (Baik, 2013; Gallo, 2019). Dopamine receptor D2 (DRD2) is a member of the D2-like receptor family, along with D3 and D4. DRD2 is expressed both pre- and postsynaptically in various brain regions, such as the striatum, midbrain, cortex, and olfactory bulb (Gallo, 2019; Mishra et al., 2018). The main function of DRD2 is to modulate dopamine synthesis and release through the intracellular inhibition of cAMP (Baik, 2013). In the striatum, DRD2 mediates the actions of dopamine that control both movement and reward-seeking (Gallo, 2019).

Excessive energy consumption and DIO may be contributed in part to dysregulated dopamine metabolism and food reward systems in the brain (Berridge et al., 2010). Reduced DRD2 activity or expression is often, but not always, associated with DIO (Baik, 2013). For example, an imaging study in men and women discovered that

striatal DRD2 receptor availability was reduced in obese individuals compared to controls, and that BMI was negatively correlated with DRD2 concentration (Wang et al., 2001). In corroboration using similar test methods, striatal DRD2 receptor availability was reduced in obese women compared to controls (van de Giessen et al., 2014). However, another imaging study in obese women found no change in DRD2 receptor availability when examining several brain regions, including the striatum, and found no correlation between DRD2 and BMI (Karlsson et al., 2015). In a study using male Sprague-Dawley rats fed a HFD (32% kcal fat) for eight weeks, there was a 42% decrease in DRD2 density in the dorsal striatum (Narayanaswami et al., 2013). Furthermore, when male and female offspring of female B6J mice bred with D2J males were fed a HFD (60% kcal fat) for 12 weeks, both males and females showed a downregulation of DRD2 mRNA in the NAc core (Carlin et al., 2013).

In contrast to these reports, there is also evidence that DRD2 may be upregulated due to DIO. In a study using male B6 mice fed a HFD (40% kcal fat) for 20 weeks, there was an induction of DRD2 mRNA in the NAc core for mice fed a HFD, and a positive correlation between final body weight and DRD2 gene expression in this brain region (Huang et al., 2005b). Another study using male B6 mice fed a HFD (58% kcal fat) for 12 weeks showed that mice fed a HFD had increased DRD2 protein expression in the NAc (Sharma and Fulton, 2013). Furthermore, when male B6 mice were fed a HFD (40% kcal fat) for only 20 days, the DRD2 binding density was increased in the dorsal and ventral striatum (South and Huang, 2008). Interestingly, a human imaging study

demonstrated that DRD2 binding potential was positively correlated to BMI in the dorsal striatum, yet negatively correlated to BMI in the ventral striatum (Guo et al., 2014). Collectively, these studies provide evidence that DRD2 dysregulation is involved as either a cause or consequence of DIO, however the direction of DRD2 alteration and pathophysiological mechanisms are poorly understood. Furthermore, the ability to measure the precise location of DRD2 dysregulation within specific brain regions and cell compartments would be ideal for elucidating these neurophysiological mechanisms.

Conclusion

The impact of DIO on neurobiology dysregulation and behavior changes has been described in the current literature. There is evidence that DIO can alter gene expression and trace element homeostasis in the brain. Furthermore, DIO can disrupt dopamine biology and can lead to unfavorable behaviors such as a reduced physical activity, anxiety, and compromised memory. Sex and genetic factors that influence these DIO-associated alterations in the brain are not well understood. Further research is needed to elucidate the influence of sex and genetic factors on neurobiological and behavioral disturbances induced by an energy dense diet and a state of DIO. The development of future treatment and rehabilitation programs could become more effective when accounting for fundamental differences in sex and genetics.

CHAPTER III

THE INFLUENCE OF SEX AND STRAIN ON TRACE ELEMENT DYSREGULATION AND GENE EXPRESSION ALTERATIONS IN THE BRAIN DUE TO DIET-INDUCED OBESITY

Abstract

Diet-induced obesity (DIO) can disrupt trace element homeostasis and gene expression in the brain, increasing the risk for neurodegeneration. Genetics and sex are biological factors that can influence these disruptions. The aim of this study was to identify main effects and interaction effects between diet, sex, and mouse strain on trace element dysregulation and gene expression due to DIO. Male and female C57BL/6J (B6J) and DBA/2J (D2J) mice were fed either a low fat diet (10% kcal from fat) or a high fat diet (60% kcal from fat) for 16 weeks, then assessed for gene expression patterns and trace element concentrations in four brain regions. In the striatum, iron was significantly elevated in B6J female mice and ceruloplasmin mRNA was significantly upregulated in D2J female mice due to DIO. In the hippocampus, zinc was substantially increased in D2J males fed a high fat diet, but substantially decreased in D2J females fed a high fat diet. There was also a dramatic induction of divalent metal transporter 1, alpha synuclein, and amyloid precursor protein for the B6J strain in this brain region due to DIO. In the olfactory bulb, there was a significant elevation of iron and manganese in male B6J mice, and an upregulation of divalent metal transporter 1, amyloid precursor protein, and alpha synuclein mRNA in male D2J mice. In the midbrain, copper was

depleted in D2J males and females fed a high fat diet. In summary, we found that the disruption of trace element homeostasis and gene expression due to DIO was brain-region dependent, and was highly influenced by sex and strain. These results emphasize the importance of considering sex and genetics as biological factors when investigating potential associations between DIO and neurodegenerative disease.

Introduction

The World Health Organization defines obesity as abnormal or excessive fat accumulation that may impair health. Approximately 13% of the global adult population was obese in 2016, and worldwide prevalence of obesity has nearly tripled since 1975 (“WHO | Obesity”). Biochemical consequences of obesity include inflammation, oxidative stress, and mitochondrial dysfunction, all of which are common pathologies of neurodegenerative disease (ND) (Mazon et al., 2017). Increasing evidence implicates obesity as a risk factor for two of the most common forms of ND: Alzheimer’s disease and Parkinson’s disease (Martin-Jiménez et al., 2017; Mazon et al., 2017). The risk of developing a ND depends on a combination of genetics and the environment (Brown et al., 2005). Our lab seeks to determine the mechanisms by which environmental factors, such as diet-induced obesity (DIO), can lead to ND.

Dysregulation of trace element homeostasis (Ferreira and Gahl, 2017) and gene expression (Hwang et al., 2017) in the brain is associated with neurodegeneration. For example, iron (Fe) accumulation and copper (Cu) deficiency are commonly found in the substantia nigra *pars compacta* of Parkinson’s disease patients (Genoud et al., 2017).

Disruptions in Fe and manganese (Mn) homeostasis can lead to several NDs, such as Parkinson's disease and Huntington's disease (P. Chen et al., 2019). Trace elements Fe, Cu, and Mn share some common transporters and enzymes, such as divalent metal transporter 1 (DMT1) and ceruloplasmin, allowing their uptake mechanisms to interact (Skjørringe et al., 2012; Ye et al., 2017). Therefore, a disruption in homeostasis of one element can impact the other elements, leading to potentially detrimental effects on the brain (Herrera et al., 2014; Skjørringe et al., 2012; Ye et al., 2017). Disruptions in gene expression are also evident in ND, such as the upregulation of amyloid precursor protein (APP) in Alzheimer's disease (Roher et al., 2017), and induction of alpha synuclein in Parkinson's disease (Tagliafierro and Chiba-Falek, 2016). Interestingly, it has been discovered that alpha synuclein accumulates in the olfactory bulb long before it does in the substantia nigra, and is associated with the loss of olfactory sense (Adler and Beach, 2016). Loss of olfaction is an early symptom of Alzheimer's disease (Attems et al., 2014) and Parkinson's disease (Fullard et al., 2017), and usually occurs years before disease onset. While trace element homeostasis and gene expression in the context of ND have been studied extensively in the substantia nigra (Dusek et al., 2015; Friedman and Galazka-Friedman, 2012), hippocampus (Fjell et al., 2014), and striatum (Mezzaroba et al., 2019) using human and animal models, it has not received the same attention in the olfactory bulb. Therefore, we included an examination of the olfactory bulb in this study, along with other Fe-rich brain regions.

Our lab recently published two articles related to the effects of DIO on Fe dysregulation and gene expression in Fe-rich brain regions in C57BL/6J (B6J) male mice (Han et al., 2019; Liu et al., 2016). DIO significantly reduced Fe in the striatum, but had no impact on the hippocampus or midbrain for mice fed a high fat diet (HFD) in one study (Liu et al., 2016). In another study, DIO resulted in elevated Fe and upregulated alpha synuclein in the midbrain, with no effect in hippocampus or striatum (Han et al., 2019). In the present study, we evaluated multiple trace elements in the context of DIO, including Fe, Mn, Cu, and Zn. Additionally, we assessed gene expression of mRNA transcripts for proteins involved in trace element homeostasis, such as DMT1 and ceruloplasmin (Lu et al., 2017), and proteins that have been implicated in the development of ND, such as alpha synuclein (Bridi and Hirth, 2018) and amyloid precursor protein (Roher et al., 2017).

The influence of sex and genetics on trace element dysregulation and gene expression has been reported in recent literature. For example, when B6J and D2J mice were exposed to paraquat, the B6J strain had elevated Fe in the midbrain and more differentially expressed genes compared to the D2J strain (Yin et al., 2011). A DIO study using female B6J and D2J mice revealed distinct trends in gene expression fed a HFD, with an upregulation of glutathione peroxidase I in B6J mice and a downregulation of glutathione reductase in D2J mice (Norris et al., 2016). A study that examined neurodegeneration and brain connectivity in a group of suspected Alzheimer's disease patients found that the effect of obesity on brain metabolism was more significant in

females versus males (Malpetti et al., 2018). Differences in gene expression between males and females can impact susceptibility to disease in a sex-dependent manner (Torres-Rojas and Jones, 2018), which is important to recognize when studying the biochemical mechanisms of ND development. To address these important sex and genetic factors, our obesity model will include male and female B6J and D2J mouse strains. These widely used inbred strains are commonly studied based on their defined genetic profiles and unique response to environmental stress (Jellen et al., 2012; Yin et al., 2011), and have proven to be appropriate models for DIO (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992).

The objective of this study was to identify interaction effects between diet, sex, and strain on trace element dysregulation and gene expression due to DIO in specific brain regions. Based on a pilot study of trace element dysregulation in B6J and D2J mice in our lab (results not published), we hypothesized that the influence of sex on DIO would impact the B6J strain more than the D2J strain, and trace element dysregulation would be region-specific. We also hypothesized that DIO would cause increases in Fe, zinc (Zn), and alpha synuclein gene expression in the olfactory bulb, as these physiological changes have been implicated in the pathogenesis of Parkinson's and Alzheimer's disease (Adler and Beach, 2016; Gardner et al., 2017; Samudralwar et al., 1995). To test these hypotheses, we evaluated gene expression and trace elements Fe, Mn, Cu, and Zn in the following brain regions due to their importance to trace element neurobiology: hippocampus, midbrain, striatum, and olfactory bulb. The specific genes

evaluated in our study were selected based on their role in trace element regulation in the brain and their potential connection to neurodegeneration.

Materials and Methods

Animals and Diet

A total of 72 male and female mice from the C57BL/6J (B6J) and DBA/2J (D2J) strains were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at post-natal day 21. After a three-day acclimation period in the animal care facility, mice were randomly assigned a control low fat diet (LFD) with 10% kcal fat/g (Research Diets, D12450J) or mineral-matched high fat diet (HFD) with 60% kcal fat/g (Research Diets, D12492) for 16 weeks (see Supplementary Table A1). Previously, it was established that a diet high in fat is successful for inducing obesity in both the B6J and D2J strains (Hu et al., 2018). Similarity in trace element content for each diet was confirmed using graphite furnace atomic absorption spectrometry (GFAAS) (Model AA240, Agilent Technologies Inc, USA). Each treatment group comprised n=9 (Table 3.1).

Determination of the number of animals required for each experiment in this study was based on a power analysis using an estimated variance from preliminary studies from our laboratory. *Ad libitum* feeding of the assigned diets was provided with free access to deionized water 24 hours/day. For the duration of the 16-week treatment, mice were weighed once per week, and food weight was recorded three days/week. Mice were housed three per cage with males and females stationed on opposite sides of a temperature-controlled room (25°C) maintained on a 12-hour light/dark cycle.

This study was conducted in an American Association for Laboratory Animal Care accredited facility following a protocol approved by the Institution of Animal Care and Use Committee at the University of North Carolina Greensboro. Procedures were performed by the principles and guidelines established by the National Institutes of Health for the ethical care and use of laboratory animals. One mouse was humanely euthanized during week 10 of the diet treatment due to lack of movement and food consumption.

Table 3.1. Study Design Based on Strain, Sex, and Diet

Diet	B6J		D2J	
	Male	Female	Male	Female
LFD	9	9	9	9
HFD	9	9	9	9

Tissue Collection

At the end of the 16-week dietary treatment, 44 mice were humanely anesthetized with isoflurane followed by rapid decapitation. The remaining mice were reserved for a separate behavioral study (data reported elsewhere). Brains were dissected sagittally into right and left hemispheres on an ice-cold stainless-steel platform into the following brain regions: olfactory bulb, hippocampus, midbrain, and striatum. Brain tissues were snap frozen in liquid nitrogen, placed on dry ice, then stored in the -80°C freezer until further processing. Right and left hemispheres were randomly assigned for analysis of trace element analysis or mRNA gene expression.

Trace Element Analysis

Trace elements Fe, Cu, Mn, and Zn were evaluated using GFAAS with concentrations reported as micrograms of metal per gram of protein. A sample size of n=5 per group was used based on a power analysis from our previous studies (Han et al., 2019; Liu et al., 2016). Total protein was analyzed using a Pierce™ bicinchoninic acid (BCA) protein quantitation assay (Thermo Fisher Scientific, Inc., USA). Brain samples were sonicated in cold radioimmunoprecipitation assay buffer (RIPA) containing protease inhibitors. Homogenates were digested in ultrapure nitric acid as a 1:1 ratio for 24 hours in a sand bath between 60-80°C. Aliquots were diluted with 2% nitric acid for use on the GFAAS. Bovine liver (NBS standard reference material, USDC, Washington DC, USA) digested in ultrapure nitric acid was used as an internal standard.

RNA Isolation and cDNA Synthesis

RNA was isolated from frozen tissue (n=3-5 per group) with the RNeasy® Plus Mini Kit (Qiagen Inc., Germantown, MD, USA) following manufacturer's protocol. RNA concentration and purity were confirmed with a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). Reverse transcription of RNA was performed on Applied Biosystems GeneAmp® PCR System 9700 using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) to prepare 20 µL samples for the thermocycler. Reaction conditions were as follows: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for five seconds, and 4°C holding

temperature at completion. Samples were stored at -20°C until further evaluation.

Specific mRNA transcripts measured in this study are listed in Table 3.2.

Table 3.2. mRNA Transcripts Related to Trace Elements and Neurodegeneration

mRNA Transcript	mRNA Abbreviation	Gene
Divalent Metal Transporter 1	DMT1	<i>SLC11A2</i>
Iron Regulatory Protein 1	IRP1	<i>ACO1</i>
Ceruloplasmin	Cp	<i>CP</i>
Copper Transporter 1	CTR1	<i>SLC31A1</i>
Alpha Synuclein	aSyn	<i>SNCA</i>
Amyloid Precursor Protein	APP	<i>APP</i>

Genes were selected for evaluation based on their relevance to the neurobiology of Fe, Mn, Cu, and Zn and their implication in neurodegeneration.

Real Time Polymerase Chain Reaction (RT-PCR)

Relative gene expression was determined by RT-PCR on a 7500 Fast Real-Time PCR System from Applied Biosystems under the following conditions: incubation for two minutes at 50°C, polymerase activation for two minutes at 95°C, and 40 cycles of PCR (denature for three seconds at 95°C and anneal/extend for 30 seconds at 95°C). Gene assays were supplied from Life Technologies (Carlsbad, CA, USA) and are listed in Table 3.2. Each assay was prepared for RT-PCR using Applied Biosystems™ Taqman™ Fast Advanced Master Mix. Expression of each gene was normalized using 18S as the endogenous control. Normalized cycle threshold (Ct) values were used to determine interactions and main effects of diet, sex, and strain. The comparative Ct method was used to determine fold change in gene expression comparing LFD (control) to HFD for each sex and strain, or to compare males (control) to females.

Statistical Analysis

The effects of diet, sex, and strain on trace element concentration and gene expression were assessed using a three-way analysis of variance (ANOVA). Statistically significant interaction effects were evaluated further for simple main effects. In cases of no interactions, statistically significant main effects are reported. Differences between treatment groups at each level were determined by pairwise comparisons with a Bonferroni adjustment applied. Normality and homogeneity of variance of data were confirmed using the Shapiro-Wilk test and Levene's test respectively. Pearson correlations were used to determine relationships between final body weight and trace element concentrations or gene expression. If equal variances could not be achieved to perform a three factor ANOVA, independent t tests were used to compare differences between groups. Statistical significance was accepted at $p < 0.05$ and differences were considered approaching significance between $p = 0.05 - 0.10$. Data are reported as means \pm standard error of the mean (SEM). Statistical analysis was performed using IBM SPSS Statistics 26.

Results

A HFD Causes Significant Weight Gain in Male and Female B6J and D2J Mice

The 16-week HFD treatment led to significant weight gain for both strains and sexes. At the start of the dietary treatment (approximately three weeks old), there were no significant differences between body weight when comparing the LFD group and the HFD group for each sex and strain (Table 3.3). By the end of the 16-week diet

treatment (approximately 18 weeks old), there was a significant difference between body weight when comparing the same groups (Table 3.3). Since there are natural body weight differences between the two sexes and strains, independent t tests were used for this comparison between diet treatment groups.

Table 3.3. Initial and Final Body Weight

Strain/Sex	Initial Body Weight (3 weeks old)			Final Body Weight (18 weeks old)		
	LFD	HFD	Significance	LFD	HFD	Significance
BM	14.30 ± 0.32	14.37 ± 0.54	p = 0.917	28.72 ± 0.41	48.16 ± 0.36	p < 0.0001
BF	13.38 ± 0.38	12.80 ± 0.26	p = 0.231	21.58 ± 0.21	41.16 ± 1.38	p < 0.0001
DM	13.14 ± 0.57	13.24 ± 0.38	p = 0.855	28.14 ± 0.78	43.91 ± 1.81	p < 0.0001
DF	12.38 ± 0.30	11.51 ± 0.37	p = 0.089	23.26 ± 0.75	34.53 ± 1.45	p < 0.0001

Body weight comparisons are between LFD groups and HFD groups for male and female B6J and D2J mice at the beginning and end of the 16-week diet treatment. Data are represented as mean ± SEM. Weight is in units of grams. BM = B6J males, BF = B6J females, DM = D2J males, DF = D2J females.

Changes in body weight over the full 16-week diet treatment for each strain and sex are shown in Figure 3.1. The percent weight gain over the 16-week diet treatment was significantly higher for mice in the HFD treatment groups compared to the LFD treatment groups for each strain and sex. Since equal variances could not be achieved to run a three-factor ANOVA, comparisons between week-1 weight and week-16 weight were made using independent t tests. B6J males fed a LFD gained 102% ± 17% grams in weight while the HFD group gained 246% ± 26% ($t_{15}=14.019$, $p < 0.0001$). For the B6J females, mice fed a LFD gained 62% ± 12% weight and mice fed a HFD gained 225% ± 32% weight ($t_{14}=14.211$, $p < 0.0001$). D2J males fed a LFD gained 117% ± 27% weight while the HFD group gained 235% ± 27% weight ($t_{15}=8.951$, $p < 0.0001$). The D2J female group fed a LFD gained 88% ± 17% weight and the HFD group gained 181% ± 37% weight

($t_{14}=6.693$, $p < 0.0001$). There was no significant difference between grams of food eaten when comparing the LFD and HFD groups, indicating that any alterations in trace element concentration would not be due to differences in dietary intake.

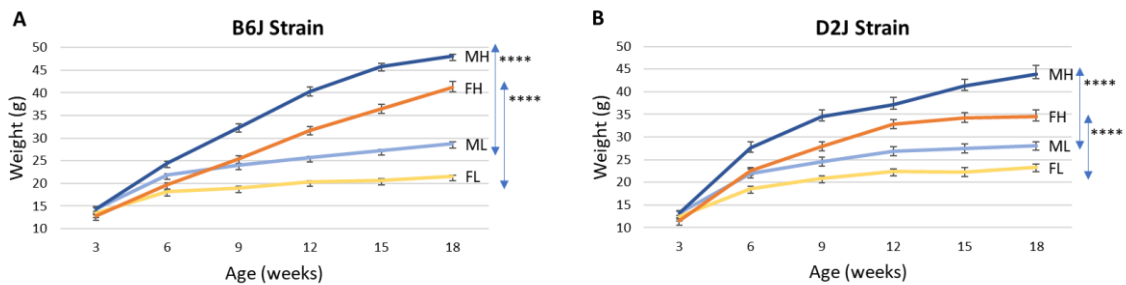


Figure 3.1. Weight Gain by Strain. Weight gain for B6J mice (A) and D2J mice (B) throughout the diet treatment. Letter codes are as follows: M=male, F=female, L=low fat diet, H=high fat diet. Data are represented as mean \pm SEM. **** $p < 0.0001$. Statistical significance refers to between-group differences.

Female Mice Fed a HFD Have Increased Fe in the Striatum

There was a significant interaction between diet and sex on Fe in the striatum ($F_{1,16} = 4.584$, $p = 0.048$), with a simple main effect of diet for female mice ($F_{1,16} = 8.140$, $p = 0.012$), but not for males ($F_{1,16} = 0.031$, $p = 0.863$) (Figure 3.2A). Female mice (B6J and D2J combined) fed a HFD show a 27% increase in Fe. Pairwise diet comparisons for each strain and sex showed a significant increase in striatum iron by 27% for B6J female mice ($F_{1,16} = 7.064$, $p = 0.017$) and a 20% increase for D2J mice, although not statistically significant for D2J mice ($F_{1,16} = 1.896$, $p = 0.188$) (Figure 3.2B).

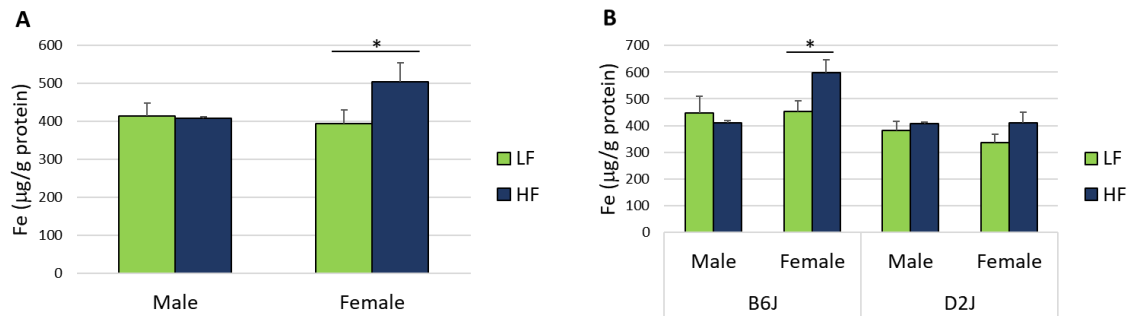


Figure 3.2. The Effect of HFD on Striatum Fe in B6J and D2J Male and Female Mice. A diet by sex interaction (A) and pairwise diet comparisons (B) both show an increase in striatum Fe in females. Data are represented as mean \pm SEM. * $p < 0.05$.

There were no statistically significant differences in Mn, Cu, or Zn in the striatum due to DIO. However, a 20% increase in Mn in female B6J mice was approaching significance ($F_{1,32} = 2.845$, $p = 0.101$). Trace element concentrations for all treatment groups in this study can be found in the appendix as Supplementary Table A2.

Midbrain Cu Decreases in D2J Mice Fed a HFD

There was a significant main effect of diet on Cu in the midbrain ($F_{1,30} = 13.645$, $p = 0.001$). When fed a HFD, D2J males had a 48% reduction in Cu compared to the LFD group ($F_{1,30} = 7.759$, $p = 0.009$), and D2J females had a 37% reduction in Cu compared to the control group ($F_{1,30} = 5.139$, $p = 0.031$) (Figure 3.3A). Although diet did not have a significant impact on Fe, Mn, and Zn in the midbrain, the D2J strain had 24% higher Mn compared to the B6J strain ($F_{1,32} = 6.380$, $p = 0.017$) (Figure 3.3B).

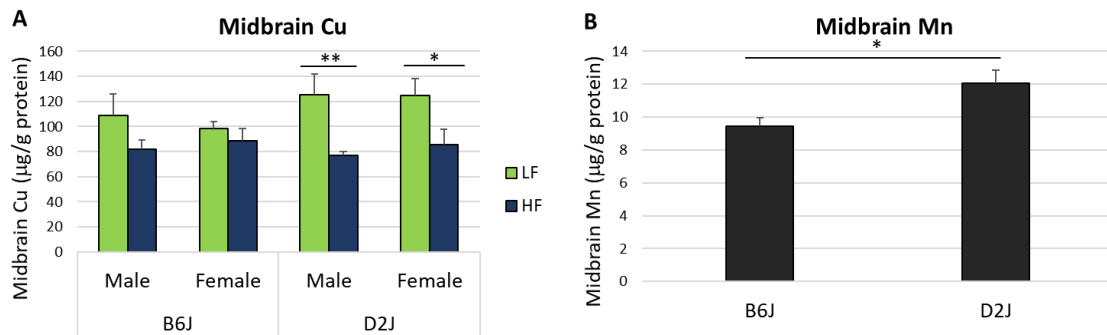


Figure 3.3. Midbrain Cu and Mn Concentrations in B6J and D2J Mice. Midbrain Cu was reduced in male and female B6J and D2J mice fed a HFD (A). The effect of strain on midbrain Mn (B) shows elevated Mn levels in the D2J strain compared to the B6J strain. Data are represented as mean \pm SEM. * p <0.05, ** p <0.01.

B6J Mice Fed a HFD Have Increased Fe, Mn, Cu, and Zn in the Hippocampus, While D2J Mice Fed a HFD Show Opposite Trends in Zn Alterations

For the B6J strain, there was an overall trend of increasing Fe, Cu, Mn, and Zn concentration in the hippocampus for mice fed a HFD (Figure 3.4A-D), with a substantial increase in Cu by 39% in B6J female mice ($F_{1,29} = 9.192$, $p=0.005$) (Figure 3.4C). Additionally, there was a diet by strain interaction on Cu ($F_{1,29} = 6.704$, $p=0.015$) which showed a 27% increase in Cu for B6J mice fed a HFD compared to the control LFD ($F_{1,29} = 7.056$, $p=0.013$), and an 11% decrease in Cu for D2J mice fed a HFD, although not statistically significant ($F_{1,29} = 1.059$, $p=0.312$). There was a three-way interaction effect between diet, sex, and strain on Zn in the hippocampus ($F_{1,28} = 9.849$, $p=0.004$), which comprised a significant simple two-way interaction between diet and sex for the D2J strain ($F_{1,28} = 12.281$, $p=0.002$), but not for the B6J strain. The simple main effects from this interaction show a significant 48% increase in Zn for D2J males fed a HFD ($F_{1,28} =$

5.443, $p=0.027$) and a significant 44% decrease in Zn for D2J females fed a HFD ($F_{1,28} = 7.045$, $p=0.013$) (Figure 3.4D).

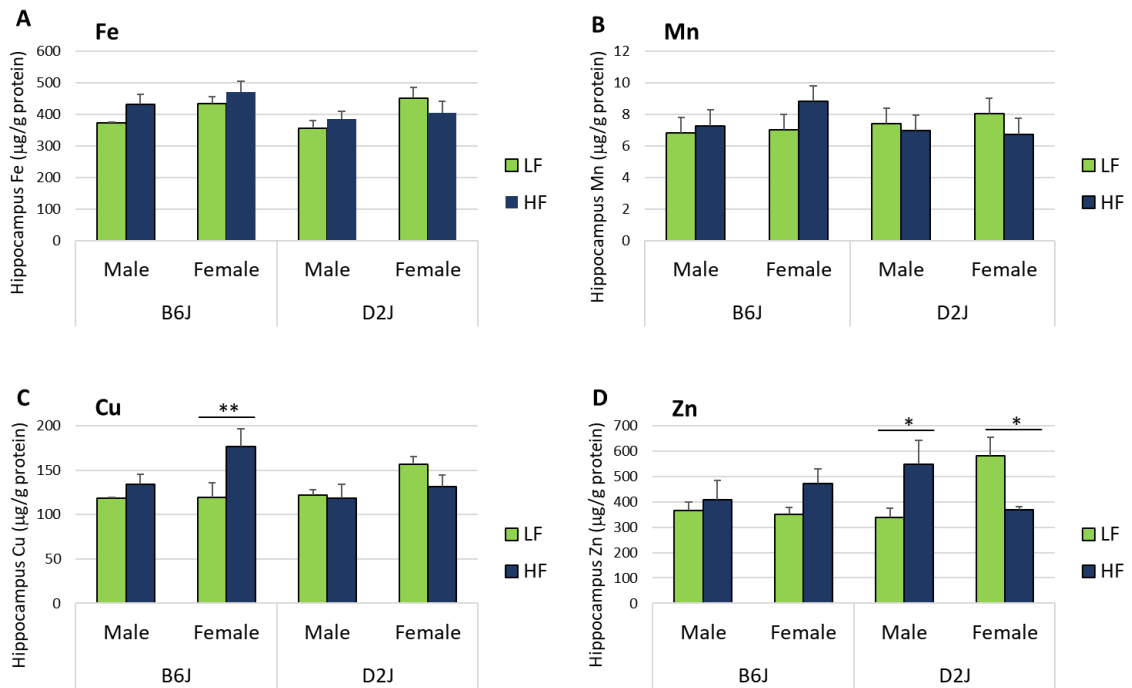


Figure 3.4. Hippocampus Trace Elements. The effect of diet, sex, and strain on hippocampus Fe (A), Mn (B), Cu (C), and Zn (D). Data are represented as mean \pm SEM. * $p<0.05$, ** $p<0.01$

It was also discovered that female mice have higher levels of Fe and Cu in the hippocampus, regardless of strain or diet. Specifically, female mice had 13% higher levels of Fe ($F_{1,30} = 6.750$, $p=0.014$) and 16% higher levels of Cu ($F_{1,29} = 5.384$, $p=0.028$) in this brain region compared to male mice.

Male B6J Mice Fed a HFD Have Increased Fe and Mn in the Olfactory Bulb

There was a significant two-way interaction between diet and sex on Fe in the olfactory bulb ($F_{1,29} = 6.241$, $p=0.018$), with a simple main effect of diet in male mice only ($F_{1,29} = 9.169$, $p=0.005$). Male mice (B6J and D2J combined) fed a HFD diet had an overall increase in Fe by 41% while female mice showed a decrease in Fe by 6%. The impact of diet on Fe in this brain region was greatest for B6J male mice, with an estimated 75% increase in Fe ($F_{1,29} = 12.987$, $p=0.001$) (Figure 3.5A). There were no interaction effects for Mn in the olfactory bulb, however, there was a statistically significant increase in Mn by 50% in B6J male mice fed a HFD ($F_{1,30} = 4.675$, $p=0.039$) (Figure 3.5B). Cu in the olfactory bulb was not significantly impacted by DIO. However, the Cu/Zn ratio in B6J females was 24% higher in the HFD group. This result was approaching statistical significance ($F_{1,31} = 2.937$, $p=0.097$).

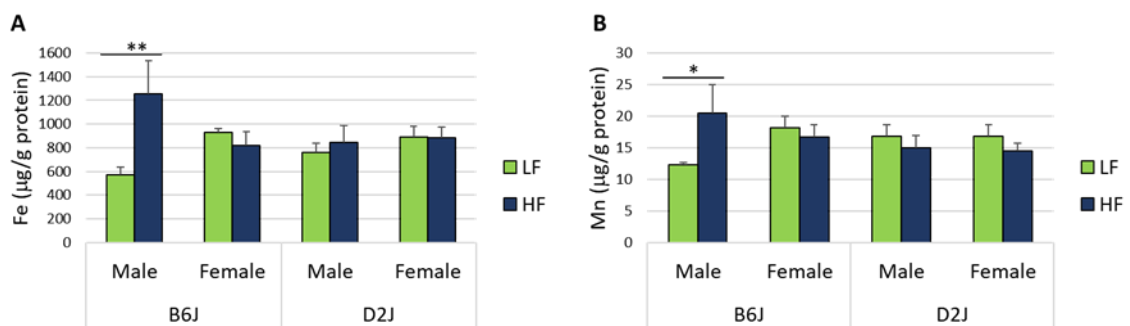


Figure 3.5. Olfactory Bulb Iron and Manganese. The effect of diet, sex, and strain on olfactory bulb Fe and Mn in male and female B6J and D2J mice. Male B6J mice fed a HFD have increased levels of both Fe (A) and Mn (B). Data are represented as mean \pm SEM. * $p<0.05$, ** $p<0.01$.

The Effect of HFD on Olfactory Bulb Zn Shows Opposite Trends by Strain and Sex

An evaluation of Zn in the olfactory bulb revealed a significant three-way interaction between diet, sex, and strain on Zn concentration ($F_{1,30} = 6.329$, $p=0.017$) (Figure 3.6). Zinc increased by 21% in B6J male mice fed a HFD, but decreased by 28% in B6J female mice. The opposite trend occurred in D2J mice, with a 26% decrease in Zn for D2J male mice fed a HFD, but a 17% increase for D2J females. While this three-way interaction was statistically significant, individual differences based on pairwise diet comparisons were not significantly different due to high variance.

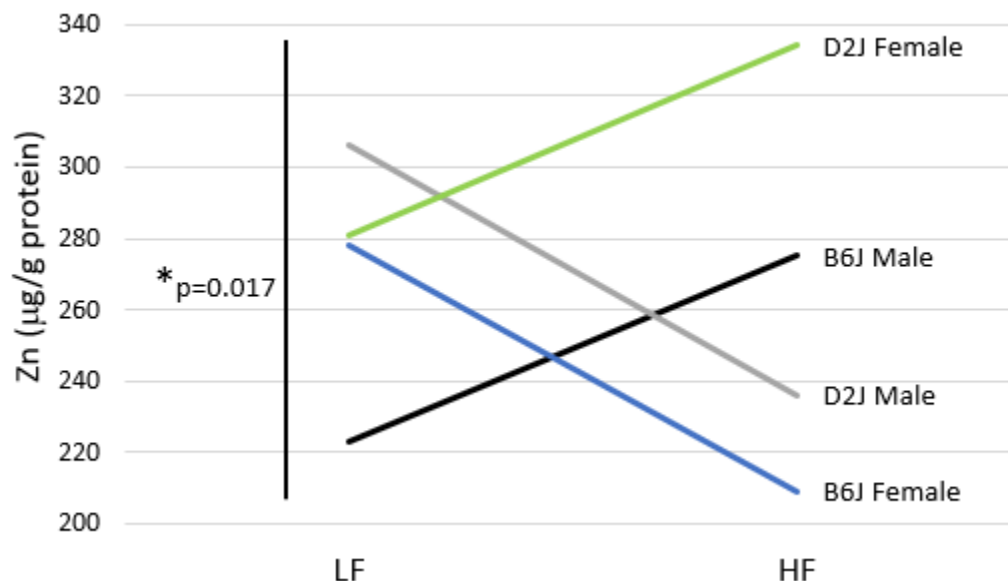


Figure 3.6. Olfactory Bulb Zinc Three-Way Interaction. Three-way interaction between diet, sex, and strain on Zn in the olfactory bulb of male and female B6J and D2J mice.

Body Weight is Correlated to Striatal Fe and Hippocampal Zn in Females

The relationship between final body weight and trace element concentration was measured using the Pearson product-moment correlation. In the striatum, there was a strong positive correlation between Fe and female body weight ($R=0.70$, $p=0.011$) (Figure 3.7A). In the hippocampus, there was a strong negative correlation between Zn and D2J female body weight ($R= -0.80$, $p=0.009$) (Figure 3.7B). The corresponding coefficient of determination (R^2) for each correlation is shown in Figure 3.7. In the striatum of female B6J and D2J mice, 49% of the variance in Fe concentration can be explained by body weight. In the hippocampus, 64% of the variance in Zn for D2J females can be explained by body weight.

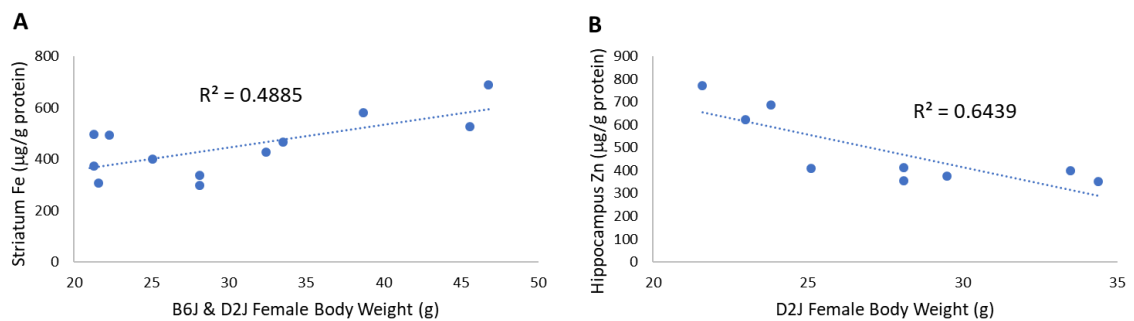


Figure 3.7. Final Body Weight and Trace Element Concentration Correlations. Body weight after the 16-week diet treatment is positively correlated to striatum Fe in female mice (A) and negatively correlated to hippocampus Zn in D2J female mice (B).

D2J Female Mice Fed a HFD Have Increased Expression of Ceruloplasmin in the Striatum

The influence of diet, sex, and strain on gene expression in the striatum was evaluated for DMT1, IRP1, ceruloplasmin, and alpha synuclein. The expression of ceruloplasmin mRNA in the striatum was significantly impacted by HFD (Figure 3.8A). Specifically, ceruloplasmin was upregulated 1.6-fold for female D2J mice fed a HFD ($F_{1,30}=6.511$, $p=0.016$) compared to mice fed a LFD. The influence of diet was not significant for B6J mice or D2J male mice. When evaluating sex and strain as factors that may influence gene expression, there was a significant sex difference in mRNA expression for DMT1, alpha synuclein, and ceruloplasmin in the striatum. Compared to B6J males, female B6J mice had 1.3-fold increased expression of DMT1 mRNA ($F_{1,30}=7.848$, $p=0.009$) and nearly 20-fold decreased expression of alpha synuclein mRNA ($F_{1,30}=916.922$, $p<0.0001$). While the sex effect was not significant in D2J mice for DMT1 and alpha synuclein, there was a significant sex effect in the D2J strain for the expression of ceruloplasmin. Compared to D2J males, ceruloplasmin expression in D2J females is 1.4-fold less ($F_{1,30}=11.685$, $p=0.002$). When examining the impact of sex by diet for ceruloplasmin, this repression was only significant for female D2J mice fed a LFD, with a 1.9-fold reduction in mRNA compared to males ($F_{1,30}=13.014$, $p=0.001$). The effect of sex on DMT1 and alpha synuclein expression was not analyzed separately by diet since there was no diet effect for either gene. IRP1 showed no significant difference in mRNA expression by diet, sex, or strain. Gene expression data for the striatum is included in the appendix as Supplementary Table A3.

DIO Significantly Impacts Gene Expression in the Hippocampus of B6J Male Mice

There were significant three-way interaction effects between diet, sex, and strain for the mRNA expression of DMT1 ($F_{1,30}=31.955$, $p<0.0001$), ceruloplasmin ($F_{1,32}=4.629$, $p=0.039$), alpha synuclein ($F_{1,28}=6.988$, $p=0.013$), and APP ($F_{1,30}=31.322$, $p<0.0001$). The impact of the HFD on the expression of these genes is shown in Figure 3.8B. For DMT1, there was an upregulation by 5-fold for B6J male mice fed a HFD ($F_{1,30}=77.306$, $p<0.0001$), but only a slight downregulation for D2J male mice, and no change in expression for females of either strain due to diet. For ceruloplasmin, there was a downregulation of mRNA expression in B6J male mice fed a HFD by 1.5-fold ($F_{1,32}=6.911$, $p=0.013$), a slight induction for D2J male mice, and no change for females. For alpha synuclein, both male and female B6J mice fed a HFD showed a significant upregulation in mRNA, with a 7-fold increase in B6J males ($F_{1,28}=41.805$, $p<0.0001$) and a 1.7-fold increase for B6J females ($F_{1,28}=4.486$, $p=0.043$). The expression of alpha synuclein in D2J mice was not impacted by the HFD. For APP, only the B6J male mice were significantly impacted by the HFD, with a 10-fold upregulation ($F_{1,30}=96.038$, $p<0.0001$). Female mice of both strains showed a slight upregulation in APP. Although diet did not have an impact on any of the mice for the expression of CTR1 (Figure 3.8B), there was a significant two-way interaction between sex and strain ($F_{1,32}=21.415$, $p<0.0001$) for the expression of this gene. The effect of strain was only significant for females, with D2J female mice showing a 2-fold decrease in expression compared to female B6J mice ($F_{1,32}=28.570$, $p<0.0001$). The effect of sex was only significant in the

D2J s, with D2J females showing a 2-fold decrease in expression compared to D2J males ($F_{1,32}=23.768$, $p<0.0001$). Gene expression data for the hippocampus is included in the appendix as Supplementary Table A4.

DIO Significantly Impacts Gene Expression in the Olfactory Bulb of D2J Male Mice

The expression of mRNA in D2J male mice was influenced by exposure to a chronic high fat diet. DMT1 mRNA expression increased by 2-fold ($F_{1,23}=4.608$, $p=0.043$), alpha synuclein was upregulated by 1.5-fold ($F_{1,22}=4.805$, $p=0.039$), and APP was upregulated by nearly 3-fold ($F_{1,23}=7.436$, $p=0.012$) (Figure 3.8C). Each gene measured in this brain region also showed a significant sex by strain interaction effect. For DMT1, the effect of sex was significant for B6J mice only ($F_{1,23}=23.708$, $p<0.0001$), with B6J females expressing 3-fold higher mRNA compared to B6J males. Alpha synuclein showed a similar pattern of a sex effect that was evident in B6J mice only ($F_{1,22}=32.455$, $p=0.0001$), with B6J females expressing 2.4-fold higher mRNA compared to B6J males. The sex by strain interaction was different for APP mRNA expression, with a significant sex effect for D2J mice only ($F_{1,23}=158.745$, $p<0.0001$). In this case, D2J males had a 23-fold higher expression level compared to D2J females. Gene expression data for the olfactory bulb is included in the appendix in Supplementary Table A5.

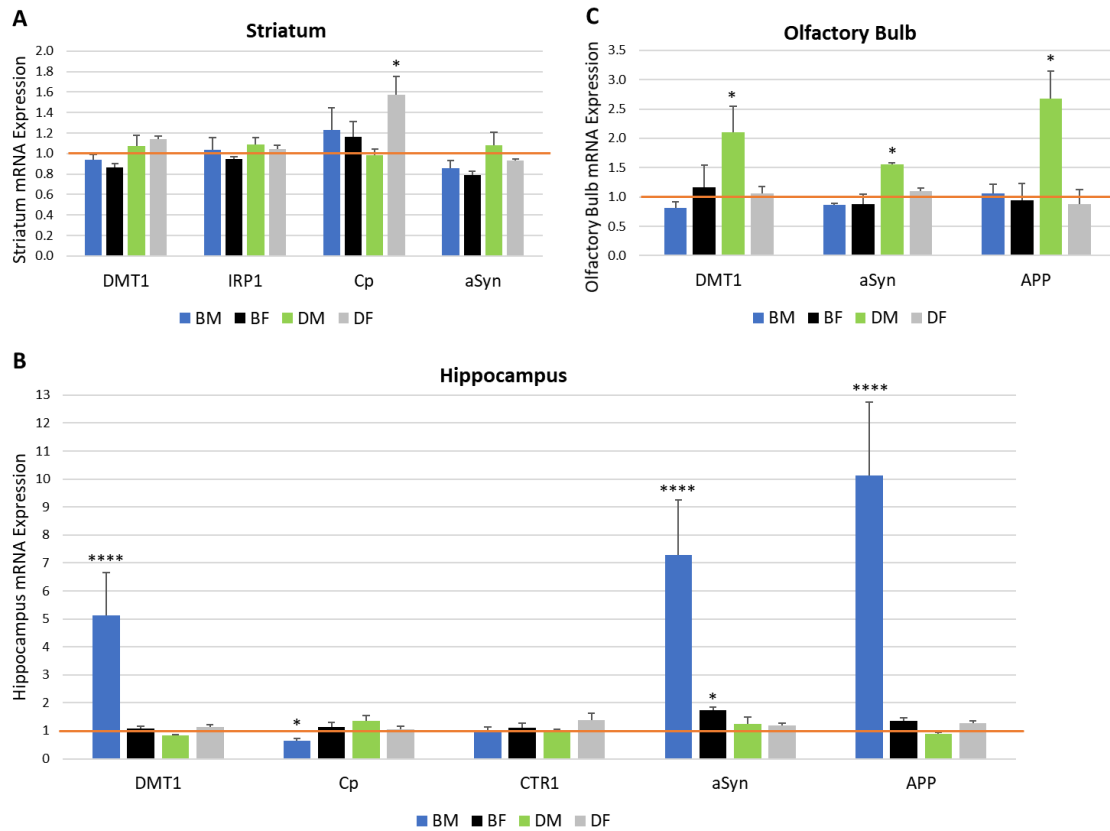


Figure 3.8. Gene Expression Related to Trace Element Regulation and ND. Fold change comparisons by diet for these genes are shown for the striatum (A), hippocampus (B), and olfactory bulb (C). The LFD mRNA expression was established as the control (set to 1). Fold change compared to each control was determined using the $2^{-\Delta\Delta Ct}$ method. Data are represented as mean \pm SEM. * $p < 0.05$, **** $p < 0.0001$. DMT1=divalent metal transporter 1, IRP1=iron regulatory protein 1, Cp=ceruloplasmin, aSyn=alpha synuclein, CTR1=copper transporter protein 1, APP = amyloid precursor protein, BM=B6J males, BF=B6J females, DM=D2J males, DF=D2J females.

Body Weight is Correlated with mRNA Expression of DMT1, Alpha Synuclein, and APP in Female D2J Mice

To understand the relationship between body weight and gene expression in the brain, Pearson correlations were evaluated using the final weight of each mouse after the 16-week diet treatment with delta Ct values from the RT-PCR experiment. Since there is an inverse relationship between delta Ct values and gene expression, positive R values indicate a decrease in mRNA expression and negative R values indicate an increase in mRNA expression. Correlation data with statistical significance for DMT1, alpha synuclein, and APP in the striatum, hippocampus, and olfactory bulb for both strains and sexes are summarized in Table 3.4. Correlations were only statistically significant for D2J females after examining scatter plots for linearity. In the striatum, DMT1 expression increases as body weight increases for D2J females ($p=0.001$). In the hippocampus, as body weight increases for D2J females, gene expression increases for DMT1 ($p=0.042$), alpha synuclein ($p=0.045$), and APP ($p=0.002$). Furthermore, in the striatum there were no significant correlations found for IRP1 or ceruloplasmin with body weight. In the hippocampus, there were no significant correlations found for CTR1 or ceruloplasmin with body weight.

Table 3.4. Correlation Between Final Body Weight and Gene Expression

Gene	Strain/Sex	Striatum		Hippocampus		Olfactory Bulb	
		R	p	R	p	R	p
DMT1	BM	0.38	0.283	-0.86	0.026	0.43	0.291
	BF	0.30	0.396	-0.04	0.922	-0.06	0.885
	DM	0.02	0.973	0.60	0.088	-0.89	0.018
	DF	-0.91	0.001	-0.65	0.042	-0.44	0.277
aSyn	BM	0.47	0.168	-0.92	0.004	0.88	0.009
	BF	0.65	0.044	-0.63	0.072	0.21	0.619
	DM	0.10	0.824	-0.17	0.658	-0.77	0.044
	DF	0.22	0.577	-0.64	0.045	-0.24	0.567
APP	BM			-0.92	0.001	-0.040	0.925
	BF			-0.58	0.080	0.149	0.726
	DM			0.33	0.382	-0.984	0.0004
	DF			-0.84	0.002	0.134	0.752

Positive correlations indicate a decrease in gene expression and negative correlations indicate an increase in gene expression. Blue text indicates no correlation due to clustering of data or nonlinear scatterplot. Red text indicates a statistically significant correlation. BM = B6J males, BF = B6J females, DM = D2J males, DF = D2J females, DMT1 = divalent metal transporter, aSyn = alpha synuclein, APP = amyloid precursor protein.

Discussion

The goal of our study was to understand the impact of DIO on trace element homeostasis and mRNA gene expression in multiple brain regions, and to identify interactions between diet, sex, and strain. Dysregulation of trace element homeostasis and gene expression in the brain has been associated with a number of NDs, such as Alzheimer's disease and Parkinson's disease (Cicero et al., 2017; Cristóvão et al., 2016; Ferreira and Gahl, 2017; Hwang et al., 2017). We have shown here that DIO disrupts Fe, Mn, Cu, and Zn concentrations and mRNA gene expression in the brain in a sex and strain-dependent fashion. Additionally, we found that obesity-induced alterations in trace element concentrations and gene expression were brain region-dependent, which

is consistent with our previous work (Han et al., 2019; Liu et al., 2016). The impact of DIO on each brain region was unique, ranging from simple cases of no effect (e.g., hippocampus Mn) to complex three-way interactions between diet, sex, and strain (e.g., olfactory bulb Zn). Although we hypothesized that the factor of sex would be more prominent in the B6J strain based on our previous pilot study, we found that sex was often a critical factor for both strains. We also hypothesized that Fe, Zn and alpha synuclein mRNA expression would increase in the olfactory bulb due to DIO. We found that Fe was in fact elevated, but for males only. The Zn outcome in this brain region depended on both sex and strain, and alpha synuclein was upregulated only in D2J males. As DIO provoked different responses in trace element homeostasis and gene expression based on sex and strain, we speculate that these biological factors are instrumental in elucidating the precise mechanisms of neurodegeneration, with potential for targeted therapeutics based on sex or genetic profile. Our research provides a snapshot of DIO effects at a particular timepoint in mice (18 weeks old), and lays the foundation for future sex and strain based DIO studies.

Effect of HFD on DIO

Our experimental design included male and female B6J and D2J mouse strains which were randomly assigned to a LFD or HFD for the duration of the study. As with our previous studies, there was a marked difference in the volume of adipose tissue while harvesting organs from our mice, with HFD mice consistently exhibiting greater volumes of fat tissue. Males and females from both the B6J and D2J strains fed a HFD

had significantly higher body weight at the end of the diet treatment (Figure 3.1). This weight gain corroborates previous reports of DIO in B6J and D2J mouse strains (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992). In contrast, there are previous reports that indicate D2J mice as responding less efficiently to a HFD, including lack of weight gain (Kirk et al., 1995), delayed weight gain (Norris et al., 2016), or similar weight gain compared to a control LFD (Funkat et al., 2004). These studies either initiated their diet treatments later than three weeks of age (typically 8-10 weeks old), or used a fat source other than lard in their HFD. Our male and female D2J mice fed a HFD all gained significantly more weight compared to mice fed a LFD, likely due to early initiation of the HFD treatment at approximately three weeks old and the incorporation of 60% lard as the source of fat. The DIO model used in our study allowed us to fully determine the influence of sex and genetics on brain regional trace element homeostasis and gene expression in the obese state.

Effect of DIO in Striatum

In the striatum, a brain region involved in controlling movement, responding to environmental stimuli (Rolls, 1994), and learning (Graybiel and Grafton, 2015), we report increased concentrations of Fe in female mice (Figure 3.2A) and upregulated gene expression of ceruloplasmin in D2J female mice (Figure 3.8A) due to DIO. To the best of our knowledge, our study is the first to show that a persistent HFD is associated with an accumulation of Fe in the female striatum. This significant increase in Fe for females strongly supports the role of DIO in the disruption of trace element homeostasis

(Figures 3.2A, 3.7A). While both the B6J and D2J female strains did show an increase in Fe, the results were more significant in the B6J strain (Figure 3.2B). Previous reports comparing C57BL/6 and DBA/2 strains have shown differences in biological response due to Fe-rich or Fe-poor diets. For example, in a study using male C57BL/6Jbg and DBA/2Jlbg strains fed a high-Fe diet for four months, the C57BL/6Jbg strain experienced increased hepatic lipid hydroperoxide levels and increased glutathione S-transferase activity compared to the DBA/2Jlbg strain (Tjalkens et al., 1998). In a study using male and female mice from the BXD recombinant inbred strains and their parental strains (B6J and D2J) fed an Fe-deficient diet for approximately 3.5 months, there was a significant diet by strain interaction on Fe concentrations in the striatum and midbrain ranging from no change to 37% change across strains (Jellen et al., 2012). In contrast to these findings in females, we found no significant change in striatum Fe status in B6J males, which is consistent with our previous work using exclusively male B6J mice (Han et al., 2019). Interestingly, it has been reported that Fe can accumulate in the striatum of multiple sclerosis patients due to inflammation of brain tissue and the attraction of Fe-rich microglia (Mezzaroba et al., 2019). During a state neuroinflammation, microglia become activated to sequester extracellular iron to protect surrounding tissues, thereby increasing intracellular iron in this mode of defense (Nnah and Wessling-Resnick, 2018). Triggers for neuroinflammation can include proinflammatory cytokines, pathogens (Nnah and Wessling-Resnick, 2018), or protein deposits such as beta amyloid (Gold and El Khoury, 2015) or alpha synuclein (Ferreira and Romero-Ramos, 2018; Q.-S. Zhang et

al., 2017). Systemically, it has been reported that DIO elicits a redistribution of Fe (increased Fe in adipose tissue and decreased Fe in liver), accompanied by upregulation of inflammatory cytokine and reduced ferromagnetic adipose tissue macrophages (Orr et al., 2014). As obesity is related to a heightened state of inflammation, it is possible that the increase in Fe that we see in our study may be related to this attraction and activation of microglia, with females being more susceptible than males for reasons that should be further investigated.

Based on these striatal Fe alterations in females, we predicted that the gene expression of DMT1 or IRP1 would be impacted by DIO in the female striatum. Although ANOVA showed no difference in mRNA expression of either gene due to DIO (Figure 3.8A), there was a significant positive correlation between body weight and DMT1 mRNA expression in D2J females (Table 3.4). Furthermore, there was a significant sex effect for the expression of DMT1, with B6J females expressing 1.3-fold higher levels of this gene compared to males. DMT1 is a ubiquitous transporter for several trace elements, including Fe, Mn, and Cu (Mezzaroba et al., 2019; Skjørringe et al., 2015). The higher expression levels of DMT1 in B6J females may explain the susceptibility of female mice to increases in striatal Fe particularly due to HFD. While the specific mechanism driving HFD-associated increased Fe is unknown, these data provide a platform to direct future studies designed to identify the mechanism(s). Our previous study examining DIO in B6J males looked at an array of Fe-regulating proteins such as hepcidin, ferroportin, transferrin receptor and ferritin and found no effect of HFD on any of them.

Due to limited tissue in the current study, we selected DMT1, IRP1, and ceruloplasmin based on their contribution to Fe homeostasis (Lu et al., 2017; Skjørringe et al., 2015; Zhou and Tan, 2017), as well as their relationship to other trace elements (Cu, Mn, and Zn) (Han et al., 2016; Jursa and Smith, 2009; Martelli and Moulis, 2004). Additionally, we evaluated alpha synuclein in the striatum, as this protein is known to aggregate with Fe during the process of neurodegeneration (B. Chen et al., 2019). Consistent with our previous work with male B6J mice fed a HFD (Han et al., 2019), alpha synuclein gene expression in males was not impacted in the striatum. Our data support that future studies looking at the impact of DIO on brain iron metabolism should include both sexes and a wider array of Fe-regulating genes to further our understanding of Fe-related neurodegenerative processes.

The gene expression of ceruloplasmin was also evaluated in the striatum. Ceruloplasmin is a Cu-dependent protein that acts as a ferroxidase, enabling cellular export of Fe into the blood via the binding to transferrin in the Fe³⁺ oxidation state (Hellman and Gitlin, 2002). This protein also acts as an oxidizing agent for Mn (Hellman and Gitlin, 2002; Jursa and Smith, 2009). Although DIO did not have an impact on the gene expression of DMT1 or IRP1 in the striatum, we found that DIO disrupted mRNA expression of ceruloplasmin in D2J female mice (Figure 3.8A), with a statistically significant upregulation by 1.6-fold. Previous studies have shown evidence of ceruloplasmin upregulation associated with neurodegenerative disease in humans and rodents. For example, postmortum brain tissue ceruloplasmin levels were elevated in

the striatum of Alzheimer's disease patients compared to young adult and elderly controls (Loeffler et al., 1996). Moreover, ceruloplasmin mRNA and protein was upregulated in the retinas of glaucomatous DBA/2 mice, but not C57BL/6 control mice (Stasi et al., 2007). In this same study, ceruloplasmin concentration was elevated in human eye tissue of patients with glaucoma. Further studies are needed to determine if the induction of ceruloplasmin that we found in the striatum of D2J females with DIO could impact Fe or Mn homeostasis with age and continued HFD feeding, or if this upregulation is associated with neurodegeneration.

In addition to the sex effect that we found on DMT1 mRNA expression, there was also a sex effect for the expression of ceruloplasmin and alpha synuclein. D2J females expressed 1.4-fold lower levels of ceruloplasmin than D2J males, and B6J females expressed 20-fold lower levels of alpha synuclein compared to male B6J mice. Collectively, these results show that the impact of DIO on trace element homeostasis and gene expression in the striatum is influenced by sex and strain. Furthermore, even when diet had no impact on biochemical parameters, the sex and strain differences that we have discovered here are important to consider when studying the pathophysiological mechanisms of ND.

Effect of DIO in Midbrain

The midbrain is of particular interest for the study of Parkinson's disease, as this brain region is known to accumulate Fe and alpha synuclein as neurodegeneration progresses (P. Chen et al., 2019; Lingor et al., 2017). Furthermore, Cu depletion in the

midbrain is a hallmark of this disease (Dusek et al., 2015; Liddell and White, 2018). In our previous work, we found that both Fe and alpha synuclein increased in the midbrain of B6J male mice fed a HFD (Han et al., 2019). In our current study using male and female B6J and D2J mice, we report that Cu was significantly reduced in male and female D2J mice, but only slightly reduced in B6J mice (Figure 3.3A). This decline in Cu is consistent with characteristics of Parkinson's disease, supporting the role of DIO as a potential trigger for trace element dysregulation and neurodegeneration. Although Cu homeostasis was altered, the mice in our study did not show significant changes in Fe, Mn, or Zn due to DIO. This may be due to the age of our mice (18 weeks old) at the time of trace element analysis, compared to our previous study that examined mice at 24 weeks old. Fe is known to accumulate in the brain with normal aging and during cases of neurodegeneration (Ward et al., 2014), and we propose that DIO can exacerbate this accumulation with time. Since mRNA concentrations were unusually low in our midbrain samples, gene expression data were limited to males only. We found that ceruloplasmin and alpha synuclein gene expression was slightly upregulated due to DIO, however these differences were not statistically significant. Gene expression data for the midbrain is included in the appendix as Supplementary Table A6. Taken together, our trace element and gene expression results show that at 18 weeks of age, B6J and D2J mice fed a HFD do not yet experience significant Fe accumulation nor alpha synuclein induction in the midbrain. Additionally, the substantial Cu reductions in D2J mice due to DIO emphasize the importance of evaluating genetics as a factor in trace

element dysregulation, and indicate that D2J mice may be impacted at an earlier age compared to the B6J strain.

Effect of DIO in Hippocampus

Several interactions between diet, sex, and strain on trace element homeostasis were discovered in the hippocampus. The hippocampus is a brain region involved in learning, memory, and cognition (Toda et al., 2019). Alterations in Cu and Zn due to DIO were more prevalent in this brain region compared to Fe and Mn. For example, female B6J mice fed a HFD had a significant 39% increase in Cu (Figure 3.4C), and there was a three-way interaction between diet, sex, and strain for Zn, affecting mainly the D2J strain (Figure 3.4D). The effect of DIO revealed a striking difference between Zn levels within the D2J strain. D2J males had a significant 48% increase in Zn, while females had a significant 44% decrease in Zn (Figure 3.4D). Additionally, D2J female body weight was negatively correlated to Zn concentration (Figure 3.7B). In the literature, there are several conflicting reports of Zn concentration in the hippocampus of Alzheimer's disease patients, with some showing increases and others reporting decreases (Panayi et al., 2002). We speculate that the sex-dependent dysregulation of Zn could be due to altered gene expression provoked by DIO. In a study that evaluated Zn-related gene expression patterns in the cortex of post mortem tissue, ZNT3 and ZNT4 mRNA expression was downregulated, while ZIP1, ZIP9, and ZIP3 were upregulated in brain tissue of obese individuals (Olesen et al., 2016). Additionally, BMI was correlated with repression of both ZNT1 and ZNT6. This study also measured the effects of sex and race,

and found that men had a higher expression of ZNT4, women had lower expression of ZIP1, and African Americans had higher expression of ZIP14 compared to Caucasians. Although these results are for cortex, it is possible that Zn dysregulation in the hippocampus may also depend on ZIP and ZNT gene expression. The activity of Cu-Zn superoxide dismutase (SOD) in postmortem brain tissue of Alzheimer's patients was also found to be sex-dependent (Schuessel et al., 2004). Cu-Zn SOD activity was higher overall in Alzheimer's brain tissue versus control tissue, but females with Alzheimer's disease had higher activity of Cu-Zn SOD than males with Alzheimer's. No sex difference in Cu-Zn SOD activity was found in the control samples. Dysregulation of Cu and Zn homeostasis is a hallmark of Alzheimer's disease, as both ions can interact with beta amyloid protein in the formation of senile plaques (Mezzaroba et al., 2019; Sensi et al., 2018), occurring mainly in the hippocampus (Cristóvão et al., 2016; Deibel et al., 1996; Tamano and Takeda, 2019). In the current study, we found a diet by strain interaction for Cu in this brain region, with B6J mice showing an increase in Cu while D2J mice showed a decrease in concentration. These strain differences suggest that genetics have a strong influence on biochemical response to DIO, and may help to explain the disparity in results regarding Cu increases or decreases reported in the literature (Mezzaroba et al., 2019). Furthermore, the opposite effects in Zn concentration for D2J males and females emphasize that sex is a critical factor when studying the impact of DIO on trace element dysregulation.

Gene expression in the hippocampus was also influenced by diet, sex, and strain. Based on our results of increased Cu levels, we speculated that there may be disturbances in CTR1 expression, a major transporter for Cu in the brain (Sharp, 2003). Contrary to our prediction, there was no impact of DIO on CTR1 expression (Figure 3.8B), however, there were significant three-way interactions between diet, sex, and strain for the mRNA expression of ceruloplasmin, DMT1, alpha synuclein, and APP. These interactions showed a greater impact on males compared to females. For example, the expression of DMT1 and ceruloplasmin had opposite effects, with DMT1 expression increasing for B6J males but decreasing for D2J males, while ceruloplasmin showed a decrease in expression for B6J males and an increase in D2J males. Expression of these two genes for females of both strains was unaffected by DIO. This leads us to infer that the increase in Cu for B6J females that we found is not directly related to the mRNA gene expression of DMT1 or ceruloplasmin. Future studies will include protein expression measurement to investigate other potential mechanisms of Cu dysregulation in B6J female mice. For alpha synuclein, there was an upregulation for both male and female B6J mice due to DIO (Figure 3.8B), but no change in D2J mice, highlighting the influence of genetics on gene expression. In the hippocampus, alpha synuclein can form protein aggregates called Lewy bodies, which are characteristic of dementia and Alzheimer's disease (Adamowicz et al., 2017). Our results suggest that the B6J strain is more susceptible to Lewy body formation in the hippocampus when fed a HFD. The effect of DIO on APP expression had the most significant impact on B6J males, with a 10-

fold increase in mRNA expression (Figure 3.8B). Amyloid precursor protein (APP) is a transmembrane protein associated with several biological functions, including cellular proliferation and differentiation, cell-fate specification, and neurite outgrowth (S. Wang et al., 2016). The overexpression of APP, however, has been implicated in the progression of Alzheimer's disease (Roher et al., 2017) since the senile plaques in this disease consist mainly of beta amyloid protein, which is derived from the proteolytic cleavage of APP. Disruptions in normal gene expression of APP can therefore lead to the potential build-up of beta amyloid, promoting the conditions for neurodegeneration. The hippocampus is particularly vulnerable to beta amyloid protein aggregation and senile plaque formation (Zhang et al., 2011). Our results are consistent with other DIO studies involving male B6J mice fed a HFD that found either beta amyloid accumulation (Busquets et al., 2017) or APP induction (Puig et al., 2012) in response to DIO.

Overall, our findings in the hippocampus demonstrate that sex and strain strongly influence the impact of DIO on Cu and Zn homeostasis and gene expression alterations in DMT1, ceruloplasmin, alpha synuclein, and APP. Sex differences were prominent in the D2J strain for Zn, while strain differences are highly pronounced for DMT1 and ceruloplasmin expression in males. Genes related to neurodegenerative pathologies, alpha synuclein and APP, show a dramatic upregulation only in male B6J mice and corroborate with previous studies in male B6 mice (Busquets et al., 2017; Puig et al., 2012). These data provide a foundation for future studies examining sex and strain influences on the effects of DIO and neurodegeneration.

Effect of DIO in Olfactory Bulb

The impact of diet, sex, and strain was also evident in the olfactory bulb, a brain region involved in the detection of odor (Nagayama et al., 2014). Compared to other brain regions, it has less protection by the blood brain barrier and more exposure to the environment through the nasal cavity, providing a potential route for toxins, or a beneficial entry for therapeutics (Crowe et al., 2018). In our study, we found a diet by sex interaction for Fe that showed an increase in Fe in males but a decrease in Fe in females due to DIO. The impact of DIO was greatest in B6J males, which showed a 75% higher concentration of Fe (Figure 3.5A) and a 50% elevation in Mn (Figure 3.5B) in the HFD group versus the LFD group. Although there were no differences in Cu concentrations in the olfactory bulb due to DIO, the Cu/Zn ratio for female B6J mice was 24% higher for the HFD group compared to the LFD group and was trending toward statistical significance ($p=0.097$). Elevated blood Cu/Zn ratios have been reported previously in cases of autism spectrum disorders (Bjørklund, 2013), aging (Giacconi et al., 2017), and inflammation (Malavolta et al., 2015). For Zn, there was a significant three-way interaction between diet, sex, and strain in the olfactory bulb (Figure 3.6). Concentrations of Zn were increased in B6J males and D2J females but were decreased in B6J females and D2J males by a similar magnitude. Interestingly, the Zn results in D2J mice in the olfactory bulb were opposite of the Zn results in the hippocampus (Figure 3.4D). It is possible that Zn may be redistributed within brain regions during obesity-induced dysregulation, causing increases in one region and decreases in others. This

highlights the importance of evaluating individual brain regions versus whole brain analysis. Our trace element results were similar in some aspects to a study using post-mortem male and female human brain tissue, which found that Fe concentration was 25% higher in Parkinson's disease olfactory bulbs compared to controls (Gardner et al., 2017). In another study using brain tissue from Alzheimer's disease patients, Fe and Zn were significantly elevated in the olfactory bulb (Samudralwar et al., 1995). Collectively, these studies demonstrate that DIO and common neurodegenerative diseases may be associated with trace element dysregulation in the olfactory bulb.

Alterations in gene expression due to DIO in the olfactory bulb were assessed for DMT1, alpha synuclein, and APP (Figure 3.8C). Considering the significant Fe and Mn alterations due to DIO found in male B6J mice, we expected to find an impact on gene expression in B6J males as well. Unexpectedly, only the D2J males were highly impacted, with a significant upregulation in all three genes due to DIO. Previous studies have also shown B6 and D2 strain differences in APP and DMT1 expression. For example, one study found that under conditions of stress, APP mRNA in the hypothalamus was upregulated in D2J mice but not in B6J mice (Tsolakidou et al., 2010). Another study found that APP protein levels were upregulated in D2J glaucomatous retinas, but not in B6 controls (Goldblum et al., 2007). In the duodenum, there were significant diet by strain interactions on the mRNA expression of DMT1 for D2 and B6 mice which showed that mRNA was upregulated more in D2 mice fed an Fe-deficient diet compared to B6 mice, while an Fe-supplemented diet resulted in a downregulation

of DMT1 in D2 mice, but had no effect on B6 mice (Dupic, 2002). In future studies, we will examine both mRNA and protein expression together to understand the downstream effects of gene expression alterations. In the current study, the increase in Fe that we found in B6J males in the olfactory bulb were not associated with an induction of alpha synuclein mRNA in B6J males. Although Fe overload is known to promote alpha synuclein aggregation (B. Chen et al., 2019), and alpha synuclein upregulation has been associated with synucleinopathies (Tagliafierro and Chiba-Falek, 2016), other studies have shown that alpha synuclein mRNA expression is unchanged or reduced in Parkinson's disease brains, with Fe inducing alpha synuclein protein synthesis at the translational level rather than the transcriptional level (Febbraro et al., 2012; Zhou and Tan, 2017). This helps to explain why we did not see an upregulation of alpha synuclein mRNA in the olfactory bulb of B6J mice fed a HFD, even though Fe was elevated in this brain region.

To summarize our results in the olfactory bulb, we found that DIO led to alterations in trace element homeostasis predominantly in male B6J mice, and gene expression dysregulation mainly in male D2J mice. While these changes in neurobiological conditions due to DIO may not be related, our results show that DIO has the potential to disrupt homeostasis by various methods in the olfactory bulb, with effects that depend on sex and genetics.

Conclusion

In conclusion, our study provides unique information regarding the impact of DIO on trace element homeostasis and mRNA gene expression in multiple brain regions, with sex and genetic factors influencing the outcome. In the striatum, Fe was significantly increased in B6J females and ceruloplasmin was significantly upregulated in D2J females. In the midbrain, there was a substantial decrease in Cu for the D2J strain only. In the hippocampus, there was a dramatic upregulation of DMT1, alpha synuclein, and APP for B6J males. Moreover, there was an opposite trend of Zn dysregulation in this brain region for the D2J strain that showed a significant increase in Zn for males but a major decrease in Zn for females. In the olfactory bulb, Fe and Mn were significantly increased for B6J males and there was a significant induction of DMT1, alpha synuclein, and APP for D2J males. A major strength of our study includes the use of two murine strains and both sexes, allowing for the evaluation of sex and genetics as biological variables. Additionally, we examined the effects of DIO in a brain region-specific manner. Limitations of our study include the lack of protein expression data and mRNA expression data for the midbrain. Although we processed the midbrain to collect mRNA, the concentrations were too low for RT-PCR analysis for most samples.

Overall, our findings provide evidence that sex and strain factors influence the effect of DIO on the brain. While we hypothesized that the effect of sex would be greater in the B6J strain, we found that sex was often a significant factor for both strains. Our hypothesis for elevated Fe levels and alpha synuclein gene expression in

the olfactory bulb was confirmed in male B6J mice and male D2J mice respectively. Consistent with our previous work (Han et al., 2019; Liu et al., 2016), we found that trace element dysregulation and gene expression alterations due to DIO were brain region-specific. The biochemical disruptions that we discovered in each brain region often differed in magnitude and direction depending on sex and strain, highlighting the important influence of sex and genetics on the impact of DIO in the brain. Considering the staggering escalation of obesity prevalence for males and females with various genetic backgrounds, these data have important health implications regarding the development of effectively tailored therapeutics.

CHAPTER IV

THE IMPACT OF SEX AND STRAIN ON THE DYSREGULATION OF BEHAVIOR, DOPAMINE BIOLOGY, AND GENE EXPRESSION IN THE BRAIN DUE TO DIET-INDUCED OBESITY

Abstract

Obesity is linked to several adverse behavioral and biochemical changes, such as reduced physical activity, increased anxiety, and compromised memory. Furthermore, diet induced obesity (DIO) can lead to gene expression alterations in the brain and dysregulated dopamine biology. The objective of our study was to determine the impact of DIO on behavior, dopamine biology, and gene expression in male and female C57BL/6J (B6J) and DBA/2J (D2J) mice. Mice were fed either a low fat diet with 10% kcal from fat or a high fat diet (HFD) with 60% kcal from fat for 16 weeks. Behavior assessments demonstrated that B6J male mice fed a HFD were impacted the most through their display of significantly reduced locomotion, reduced rate of habituation to a novel environment, lack of motivation, and elevated anxiety levels in the open field. Dopamine clearance in the dorsal striatum was significantly reduced in both male and female D2J mice due to DIO, while in the nucleus accumbens core, reductions in dopamine clearance occurred for male mice of both strains fed HFD. Our evaluation of mRNA gene expression showed that dopamine receptor D2 (DRD2) was significantly upregulated in the striatum of B6J males. In the olfactory bulb, DIO caused a significant

upregulation of DRD2 and tyrosine hydroxylase in D2J males, and an induction of brain-derived neurotrophic factor expression in B6J females. In summary, our study provides evidence for important sex and strain differences on the impact of DIO-induced behavior alterations and neurobiology dysregulation. As the incidence of obesity continues to rise worldwide, these data have key health implications related to debilitating behavior disorders that can be triggered by a HFD and a state of DIO.

Introduction

Obesity and overweight prevalence is escalating worldwide, with an estimated 39% of adults classified as obese or overweight (Chooi et al., 2019). The adverse effects of obesity have been linked to various behavioral and biochemical changes, such as reduced physical activity (Sanyaolu et al., 2019), increased anxiety (Baker et al., 2017), and compromised memory (Davidson et al., 2014). Furthermore, diet induced obesity (DIO) can lead to gene expression alterations in the brain (Gan et al., 2015; Huang et al., 2005a; Wu et al., 2017) and dysregulated dopamine biology (Leite and Ribeiro, 2019). As conditions of overweight and obesity are increasing in both males and females worldwide, these alterations in behavior and biochemistry due to obesity can have serious consequences related to declining health, reduced productivity, and increased health care costs (Trogdon et al., 2008).

It has been demonstrated that physical activity or mobility can be impacted by a state of obesity in humans and rodents. In humans, obesity has been associated with decreased fine motor control and speed (C. Wang et al., 2016), reduced functional

mobility in adults (Forhan and Gill, 2013; Trivedi et al., 2015), and decreased physical activity in children and adolescents (Sanyaolu et al., 2019). In rodents, there are mixed results. In some studies, C57BL/6J (B6J) and C57BL/6 (B6) mice fed a high fat diet (HFD) show reduced locomotion due to HFD (Almeida-Suhett et al., 2017; Krishna et al., 2016; Tsai et al., 2018; Wu et al., 2018), while other studies show no impact of diet on physical activity (Bridgewater et al., 2017; Zilkha et al., 2017). Sex differences in physical activity or locomotion in the context of obesity have also been reported. In a cross-sectional study in 964 community dwelling older adults, obese women were found to be less active than obese men (Gretebeck et al., 2017). In B6J and B6 mice, male mice fed a HFD are frequently reported as having reduced locomotion in an open field (Almeida-Suhett et al., 2017; Gelineau et al., 2017; Tsai et al., 2018; Wu et al., 2018), while female mice show mixed results, with some having decreased locomotion (Krishna et al., 2016), some increased locomotion (Krishna et al., 2015), and others with no effect (Bridgewater et al., 2017; Gelineau et al., 2017). These discrepancies may be due to the duration of dietary treatment, age of behavioral testing, and diet composition. Although the precise mechanisms of how DIO can impact mobility are not clear, it is possible that DIO may disrupt the expression of genes such as dopamine receptor D2 (DRD2) and tyrosine hydroxylase (TH), which are associated with dopamine biology and physical activity (Gallo, 2019; Jang et al., 2017). Furthermore, it is possible that DIO may disrupt dopamine release and clearance in the striatum, as this brain region is known to be a primary regulator of spontaneous physical activity (Rosenfeld, 2017).

Obesity has been associated with a higher prevalence of anxiety, as demonstrated in several human studies (Baker et al., 2017; Gariépy et al., 2010; Strine et al., 2008) and rodent studies (Almeida-Suhett et al., 2017; Krishna et al., 2016). For example, a cross-sectional study of 217,379 adults in the United States found a positive association between obesity and anxiety (Strine et al., 2008). Similarly, a study in the United States with 9125 adults found that obesity lead to an approximate 25% increase in odds of having an anxiety disorder (Simon et al., 2006). A meta-analysis in China that included 17,894 children and adolescents found a significantly higher incidence of anxiety in obese and overweight subjects (40%) compared to normal weight subjects (14%) (Wang et al., 2019). In rodent DIO studies, B6J male mice (Almeida-Suhett et al., 2017) and B6 female mice (Krishna et al., 2016) fed a HFD displayed higher anxiety-like behavior in an open field as assessed by decreased center time. Additionally, male Fischer 344 rats (Buchenauer et al., 2009) and female Long Evans rats (Sivanathan et al., 2015) fed a HFD also exhibited more anxiety-like behavior compared to normal weight rats fed a control diet. In contrast, there are other reports in humans and rodents that found no link between obesity and anxiety (Araujo et al., 2017; Gelineau et al., 2017; Tsai et al., 2018). The relationship between obesity and anxiety is complex, often due to comorbidities and a potential bidirectional association (Baker et al., 2017). More research is needed to understand the physiological mechanisms that may connect obesity to anxiety.

Research that includes both sexes in the study of DIO-impact on behavior in rodents is limited. In one study using male and female B6 mice fed a HFD for 12 weeks, males fed a HFD spent less time in the center zone of the open field compared to males fed a control diet and compared to all females, indicating increased anxiety-like behavior in males only due to HFD (Bridgewater et al., 2017). Additionally, there was a sex difference in ambulation, with male B6 mice showing reduced physical activity compared to B6 females. Interestingly, diet had no effect on locomotion for either sex. In contrast to these results, a study using male and female B6J mice fed a HFD for approximately 10 weeks found no significant difference in open field center time due to diet or sex (Gelineau et al., 2017). Moreover, male mice fed a HFD had reduced locomotion compared to the male low fat diet (LFD) group, yet there was no diet impact on female locomotion, and no overall sex differences in locomotion. In this same study, males expressed less brain-derived neurotrophic factor (BDNF) compared to females, but there was no significant impact of diet on BDNF expression for either sex. While both studies report an impact of diet or sex on various behaviors, the results are inconsistent, which may be due to genetic differences between B6 and B6J mice. Both studies initiated the HFD at a similar age (6-7 weeks), but the diet treatment duration was different (12 weeks versus 10 weeks). The anxiety-like behavior in males was revealed after the 12-week diet treatment, which could be a result of increased body weight, older age, or extended exposure to the HFD.

Obesity can also have a negative impact on memory. In human studies, a high body mass index (BMI) was associated with poor memory in adolescents (Tee et al., 2018) and adults (Coppin et al., 2014). Seniors with obesity were also found to have compromised memory when compared to a normal weight group (Clark et al., 2016). An extensive review of the impact of HFD on learning and memory in rodent studies using various test measures found that most studies, but not all, found an association between HFD or DIO and memory decline (Cordner and Tamashiro, 2015). Collectively, most of these studies show a pattern of obesity with reduced memory, but few distinguish between males and females or differences in genetics that may cause discrepancies in the results.

Dopamine plays an important role in regulating motor control, cognition, and motivation (Mishra et al., 2018). Previous DIO studies in mice have found an inverse relationship between dopamine signaling with body weight (Zilkha et al., 2017) and DIO-induced anxiety with dopamine turnover in the brain (Krishna et al., 2015). In both the dorsal and ventral striatum, the regulation of dopamine neurotransmission is implicated as a modulator of DIO and food reward (Baik, 2013). For example, in a study with male B6J mice fed a high fat/high sugar Western style diet for 16 weeks, dopamine release in the dorsal striatum was increased and dopamine clearance in the dorsal striatum was decreased in mice fed the Western style diet (Fritz et al., 2018). In the nucleus accumbens (NAc) core of the ventral striatum, dopamine release assessed by fast scan cyclic voltammetry was increased in male Sprague-Dawley rats when exposed to a

sucrose-based food reward (Roitman, 2004). In a study with male B6J mice fed a HFD for six weeks, voltammetry measurements revealed a significant decrease in dopamine reuptake in the NAc (Fordahl and Jones, 2017). The NAc of the ventral striatum is involved in the mediation of reward, satisfaction, and motivation, and has been implicated in numerous behavioral disorders, such as anxiety, obsessive-compulsive disorder, and addiction (Salgado and Kaplitt, 2015). The dorsal striatum is involved in habitual and compulsive behaviors such as food-seeking and binge eating, and plays a role in homeostatic energy consumption (Fritz et al., 2018). Both regions are important to consider when investigating dopamine biology as it relates to DIO.

DIO has been associated with gene expression dysregulation in the brain, with implications for the development of various behavior impairments, such as increased anxiety, reduced motor function, or compromised memory (Almeida-Suhett et al., 2017; Krishna et al., 2016; Lee et al., 2010). In our study, we examined the impact of DIO on the mRNA expression of BDNF, TH, and DRD2. The role of BDNF in brain plasticity is correlated with learning, memory, and cognition in humans and rodents (Miranda et al., 2019). In previous rodent studies, a HFD was associated with reduced BDNF expression in some cases (Molteni et al., 2002; Pistell et al., 2010; Wang et al., 2017), but showed an increase in expression in other cases (Gan et al., 2015; Genzer et al., 2016). TH is a rate limiting enzyme required for the synthesis of catecholamines such as dopamine, epinephrine, and norepinephrine (Daubner et al., 2011). These catecholamines serve brain functions such as attention, memory, and cognition. TH is present within the

neurons of the dopaminergic pathway that extends from the substantia nigra to the striatum, which is imperative for proper motor function (Jang et al., 2017). Obesity has been shown to have a negative impact on mobility (Forhan and Gill, 2013) and has been associated with changes in TH gene expression in different brain regions (Huang et al., 2005a; Lee et al., 2010; Ong et al., 2013; Wu et al., 2017). DRD2 is a member of the D2-like receptor family, and is expressed both pre- and postsynaptically in various brain regions, such as the striatum, midbrain, cortex, and olfactory bulb (Gallo, 2019; Mishra et al., 2018). The main function of DRD2 is to modulate dopamine synthesis and release through the intracellular inhibition of cAMP (Baik, 2013). In the striatum, DRD2 mediates the actions of dopamine that control both movement and reward-seeking (Gallo, 2019). Reduced DRD2 activity or expression has been associated with DIO in some human studies (van de Giessen et al., 2014; Wang et al., 2001) and rodent studies (Carlin et al., 2013; Narayanaswami et al., 2013). However, conflicting reports show an increase in DRD2 in mice (Huang et al., 2005a; Sharma and Fulton, 2013; South and Huang, 2008), or no change in humans (Karlsson et al., 2015). Interestingly, a human imaging study demonstrated that DRD2 binding potential was positively correlated to BMI in the dorsal striatum, yet negatively correlated to BMI in the ventral striatum (Guo et al., 2014). Collectively, these studies provide evidence that BDNF, TH, and DRD2 dysregulation may be involved as either a cause or consequence of DIO, however the direction of alteration and pathophysiological mechanisms are poorly understood.

There are very few rodent studies that address the influence of both strain and sex on behavior changes and gene expression alterations due to DIO. The aim of this study was to investigate the impact of DIO on behavior change, gene expression, and dopamine release and reuptake using male and female B6J and DBA/2J (D2J) mice as a model to examine sex and strain influences. These strains were selected based on their frequent use in behavioral neuroscience and prior studies exhibiting differential traits (Mozhui et al., 2010). B6J and D2J mice represent key strains that are represented in the Mouse Phenome Project Database (Bogue et al., 2018; Grubb et al., 2014) and are used as parental strains in the BXD recombinant inbred strain set used as genetic reference populations to evaluate genetic determinants of correlated phenotypes for the GeneNetwork open source project (Philip et al., 2010). Furthermore, these strains have been validated as appropriate models for DIO (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992). In the current study, gene expression was evaluated in the striatum, hippocampus, and olfactory bulb, and dopamine release and reuptake were assessed in the dorsal and ventral striatum. We hypothesized that DIO would have a greater impact on males compared to females, and that D2J mice would be more resistant to behavior and biochemical changes compared to the B6J strain based on previous studies (Bridgewater et al., 2017; Gelineau et al., 2017; Kuleshkaya et al., 2014; Yin et al., 2011).

Materials and Methods

Animals and Diet

Male and female mice from the C57BL/6J (B6J) (n=36) and DBA/2J (D2J) (n=36) strains were purchased from Jackson Laboratory (Bar Harbor, ME, USA) at post-natal day 21. After three days of acclimation in the animal care facility, mice were randomly assigned a control LFD with 10% kcal fat/g (Research Diets, D12450J) or mineral-matched HFD with 60% kcal fat/g (Research Diets, D12492) for 16 weeks (see Supplementary Table A1). Each diet treatment group comprised n=9, with a total of n=72. *Ad libitum* feeding of the assigned diets was provided with free access to deionized water 24 hours per day. All mice were weighed once per week during the 16-week diet treatment, and food weight was recorded three days per week. Mice were housed three per cage by strain with males and females positioned on opposite sides of a temperature-controlled room (25°C) maintained on a 12-hour light/dark cycle. Sample size for the analysis of behavior, gene expression, and voltammetry (Table 4.1) was determined based on a power analysis from previous studies in our lab.

This study was conducted in an American Association for Laboratory Animal Care accredited facility following a protocol approved by the Institution of Animal Care and Use Committee at the University of North Carolina Greensboro. Procedures were performed by the principles and guidelines established by the National Institutes of Health for the ethical care and use of laboratory animals. One mouse was humanely euthanized during week 10 of the diet treatment due to failure to thrive.

Table 4.1. Study Design for Behavior, Gene Expression, and Voltammetry

Test	B6J		D2J	
	Male	Female	Male	Female
Behavior	18	18	17	18
Gene Expression	14	10	10	10
Voltammetry	4	8	7	8

Sample size for each group was determined using a power analysis based on previous data from our lab. Behavior testing occurred during weeks 14-15 of the diet treatment. Gene expression was measured after 16 weeks of diet treatment. Voltammetry experiments were conducted over a 14-day period after the 16-week diet treatment.

Open Field

The open field test was conducted during week 14 of the diet treatment and was used to evaluate locomotion and anxiety. Our test design for open field was based on published protocols (Gellért and Varga, 2016; Seibenhener and Wooten, 2015) and current literature (Almeida-Suhett et al., 2019; Bridgewater et al., 2017; Krishna et al., 2016). Mice were acclimated to the behavior test room for a minimum of 30 minutes before each experiment. Clear acrylic 29 cm x 29 cm x 38 cm cubes covered with opaque white paper on all sides were used as the test arena. Cubes were cleaned with a disinfectant spray after each test and allowed to dry for 10 minutes before starting the next test. Recording software (TopScan Lite Version 2.00, Clever Systems, Inc.) and video camera equipment were used for each recording. There were four separate test arenas (cubes) in the behavior test room, allowing for four mice to be tested at one time (see Supplementary Figures B1 and B2). Cube assignments were rotated to prevent proximity to the door from being a confounding factor. Mice had free access to food and water in their home cages, but did not have access to food or water in the testing

arena. All open field testing was conducted between 9am-1pm. Mice were placed in the center of the cube at the start of each test, and activity was recorded for 30 minutes. At the end of each test, mice were transferred from the test cube to a separate polycarbonate cage to avoid inducing anxiety in the cage of remaining mice. Fecal boli were counted manually at the end of each test as an additional measure of anxiety.

Open field videos were analyzed using TopScan Lite Version 2.00, Clever Systems, Inc. Five-minute intervals were analyzed for each 30-minute video to measure changes in behavior over time. Data from these videos were used to evaluate differences in total distance travelled (TDT), velocity, habituation, and center entries. Rate of habituation was calculated by regressing distance travelled versus time during each five-minute interval with regression coefficients (slopes) used to determine the rate. A center zone of 30% was delineated to measure center entries (center entries are inversely proportional to anxiety-like behavior). The first five-minute time segment (0-5 minutes in the open field) was used for center entry analysis for the B6J strain, and the second five-minute time segment (5-10 minutes in the open field) was used for center entry analysis for the D2J strain. These specific time frames were selected based on the activity level of each strain.

Novel Object Recognition

The novel object recognition (NOR) test was used to evaluate memory, and was designed based on published protocols (Antunes and Biala, 2012; Paola, 2011). This test

comprised of three phases: habituation, familiarization, and testing. The open field test described above was used as the habituation phase (phase I). All NOR testing was conducted between 1pm-6pm, with each test starting approximately 24 hours after the corresponding open field test. The objects used were nonporous figures with similar size and color, but distinct shape (Supplementary Figure B3). Objects were placed five centimeters from the back and side wall of each cube (Supplementary Figure B4). Mice were placed in the cubes facing away from the objects on the opposite wall. During the familiarization phase (phase II), mice were introduced to two identical objects and allowed to explore for five minutes. Cubes and figures were cleaned with a disinfectant after each test. The testing phase (phase III) began two hours after the familiarization phase. During this last phase, one of the familiar objects was replaced with a new object. Placement of the new object was alternated between left and right side of the cube to counterbalance any preference for cube location. Mice were given five minutes to explore the new and familiar objects during this testing phase.

Videos were analyzed manually for the entire five-minute video per mouse. Hand counters were used to determine the amount of time spent exploring each object. The mouse was considered exploring the object when the nose was touching the object. Results are reported here using the discrimination index as recommended in NOR protocols (Antunes and Biala, 2012; Krishna et al., 2015). The formula used to calculate discrimination index is as follows: $(T_{\text{new}} - T_{\text{familiar}}) / (T_{\text{new}} + T_{\text{familiar}})$. Rodents are naturally curious animals and will explore new objects as a normal process. Therefore, a positive

discrimination index value indicates normal behavior (exploring the new object more than the familiar object).

Nestlet Shredding

Nestlet shredding tests were conducted during week 15 of the diet treatment for the evaluation of motivation (Nichols et al., 2016), compulsivity (Angoa-Pérez et al., 2013), and overall welfare (Gaskill et al., 2013). All mice were acclimated to the behavior test room for 30 minutes prior to assessment. Each nestlet was made from standard cotton material and measured 5.8 cm x 5.8 cm x 0.2 cm. Nestlets were acclimated to the behavior room for three days prior to testing to allow for adjustments to humidity. Each nestlet was then weighed on an analytical balance on the day that testing began. Polycarbonate mouse cages with a fitted filter-top cover were filled with fresh bedding to a depth of 0.5cm (Supplementary Figure B5). One nestlet was placed in the center of each cage. Screening dividers were placed between test cages to avoid distractions from other mice. Each mouse was placed in a cage by itself with a nestlet and allowed to shred for 30 minutes. After each test, shredded nestlet material was carefully removed from each nestlet square. Nestlets were dried for 24 hours, then reweighed to determine the degree of shredding. Results are reported here as percent nestlet shredded.

Tissue Collection

After the 16-week diet treatment, 44 mice were humanely anesthetized with isoflurane followed by rapid decapitation. Brains were dissected sagittally into right and

left hemispheres on an ice-cold stainless-steel platform. The following brain regions were isolated for this study: olfactory bulb, hippocampus, and striatum. Brain tissues were snap frozen in liquid nitrogen, placed on dry ice, then stored at -80°C. Brain tissue for the remaining 27 mice was obtained over a 14-day period after the 16-week diet treatment for voltammetry experiments (described below).

RNA Isolation and cDNA Synthesis

RNA was isolated from brain tissue samples (n=3-5 per group) with the RNeasy® Plus Mini Kit (Qiagen Inc., Germantown, MD, USA) following manufacturer's protocol. RNA concentration and purity were determined using a NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific, Inc., USA). Reverse transcription of RNA was conducted on Applied Biosystems GeneAmp® PCR System 9700 using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) to prepare 20 µL samples for the thermocycler. Reaction conditions were applied as follows: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for five seconds, and 4°C holding temperature at completion. Samples were stored at -20°C until further evaluation of relative gene expression. Specific mRNA transcripts measured in this study are listed in Table 4.2.

Table 4.2. mRNA Transcripts Related to Behavior and Dopamine

mRNA Transcript	mRNA Abbreviation	Gene
Brain derived neurotrophic factor	BDNF	<i>BDNF</i>
Dopamine receptor D2	DRD2	<i>DRD2</i>
Tyrosine hydroxylase	TH	<i>Th</i>

Genes were selected for evaluation based on their relevance to behavior and dopamine biology.

Real Time Polymerase Chain Reaction (RT-PCR)

Relative gene expression was determined by RT-PCR on a 7500 Fast Real-Time PCR System from Applied Biosystems using the following conditions: incubation for two minutes at 50°C, polymerase activation for two minutes at 95°C, and 40 cycles of PCR (denature for three seconds at 95°C and anneal/extend for 30 seconds at 95°C). Gene assays were purchased from Life Technologies (Carlsbad, CA, USA) and are listed in Table 4.2. Each assay was prepared for RT-PCR using Applied Biosystems™ Taqman™ Fast Advanced Master Mix. The endogenous control 18S was used to normalize the expression of each gene. Normalized cycle threshold (Ct) values were used to determine interactions and main effects of diet, sex, and strain. The comparative Ct method ($2^{-\Delta\Delta C_t}$) was used to determine fold change in gene expression comparing LFD (control) to HFD for each sex and strain, or to compare males (control) to females.

Dopamine and Voltammetry

Dopamine release and uptake were evaluated using ex-vivo fast scan cyclic voltammetry. This technique has been used previously to determine dopamine terminal function in mice fed a HFD (Fordahl and Jones, 2017). Male and female B6J (n=12) and D2J (n=15) mice were evaluated over a two-week time period after the 16-week diet

treatment. During this time, mice were fed their respective LFD or HFD. Mice were rendered unconscious using isoflurane, decapitated, and the brain quickly removed. The brain was sliced (300 μ m width) on the coronal plane, and slices containing the dorsal and ventral striatum were incubated in artificial cerebral spinal fluid until and throughout voltammetry recordings. The remaining brain tissue was dissected into olfactory bulb, cortex, dorsal striatum, and ventral striatum, and stored at -80°C. Carbon fiber electrodes were used to record dopamine release and pre-synaptic uptake in the following regions: dorsal striatum, NAc core, and NAc shell. Dopamine release was evoked with either a single pulse (1P) stimulation (350 μ A) to measure tonic activity or a phasic burst of five stimulations (350 μ A, each at a frequency of 20Hz) (5P) to simulate physiological firing rate of dopamine neurons. Data was recorded as the maximal rate of dopamine uptake (V_{max}), 1P dopamine release, and 5P dopamine release. The percent 5P to 1P dopamine release was calculated to provide information about the dynamic capacity to release dopamine from specific brain regions. Our lab used data from the dorsal striatum and NAc core to determine relationships among DIO-impacted dopamine biology and behavior.

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics 26. A three-factor mixed plot analysis of variance (ANOVA) for each strain was used to assess behavior changes over time, with time as the within subject factor and sex and diet as between subject factors. This test was applied to TDT and velocity. A three-factor ANOVA with

diet, sex, and strain as between subject factors was used to evaluate gene expression (with ΔC_t values), V_{max} , dopamine release, and fecal boli. Since ambulatory characteristics of each strain differed significantly, a two-factor ANOVA was used for each strain with diet and sex as between subject factors for the evaluation of habituation rate, center entries, and NOR. Statistically significant interactions were evaluated for simple main effects. If no interactions were present, significant main effects are reported. Differences between treatment groups at each level were determined by pairwise comparisons with a Bonferroni adjustment applied. Independent t tests were used to compare weight gain and weight differences between the LFD and HFD groups for each strain and sex. A Welch's test for unequal variances was used for nestlet shredding. Pearson correlations were used to determine relationships between final body weight and TDT or velocity.

Normality was confirmed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. Statistical significance was accepted at $p < 0.05$ and differences were considered approaching significance between $p = 0.05 - 0.10$. Data are reported as means \pm standard error of the mean (SEM).

Results

Mice Fed a HFD Gained a Significant Amount of Weight Compared to Mice Fed a LFD

All male and female B6J and D2J mice fed a HFD gained a significant amount of weight by the end of the 16-week diet treatment. Since there are natural body weight differences between the two sexes and strains, independent t tests were used to

compare weight gain between diet treatment groups of the same sex and strain. Before starting the diet treatment (approximately three weeks old), there were no significant differences between body weight when comparing the LFD group and the HFD group for each sex and strain. At the end of the 16-week diet treatment (approximately 18 weeks old), there was a significant difference between body weight (Table 4.3).

Table 4.3. Final Body Weight

Strain/Sex	Final Body Weight (g)		Significance
	LFD Group	HFD Group	
BM	28.72 ± 0.41	48.16 ± 0.36	p < 0.0001
BF	21.58 ± 0.21	41.16 ± 1.38	p < 0.0001
DM	28.14 ± 0.78	43.91 ± 1.81	p < 0.0001
DF	23.26 ± 0.75	34.53 ± 1.45	p < 0.0001

Body weight comparisons are between LFD groups and HFD groups for male and female B6J and D2J mice at the end of the 16-week diet treatment. Data are represented as mean ± SEM. BM = B6J males, BF = B6J females, DM = D2J males, DF = D2J females.

Changes in body weight over the 16-week diet treatment for male and female B6J and D2J mice are shown in Figure 4.1.

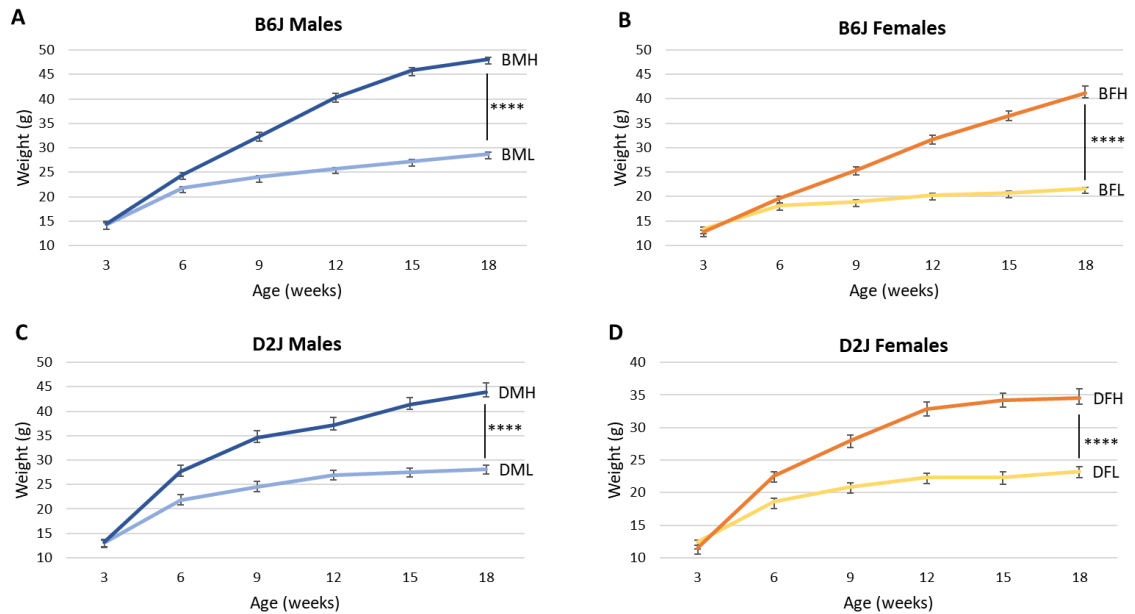


Figure 4.1. Weight Gain by Strain and Sex. Weight gain throughout the 16-week diet treatment for B6J males (A), B6J females (B), D2J males (C), and D2J females (D). Letter codes are as follows: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet. Data are represented as mean \pm SEM. **** $p < 0.0001$.

The percent weight gain for each group over the 16-week diet treatment was described previously in a companion study. Briefly, percent weight gain was significantly higher for mice in the HFD treatment groups compared to the LFD treatment groups for each strain and sex ($p < 0.0001$ for each comparison: CML vs CMH, CFL vs CFH, DML vs DMF, DFL vs DFH). There was no significant difference between grams of food eaten when comparing the LFD and HFD groups, indicating that all groups consumed similar levels of vitamins and minerals in the diet.

B6J and D2J Mice Fed a HFD Travel Less Distance in the Open Field

A three-factor mixed ANOVA was used to evaluate the effects of time, diet, and sex on TDT, with diet and sex as the between subject factors and time as the within subject factor. B6J and D2J strains were analyzed separately due to differences in ambulation. Distance traveled was analyzed in six 5-minute time intervals for a total of 30 minutes. In the repeated measures within-subject analysis, there was a statistically significant two-way interaction between time and sex in B6J mice ($F_{5,160}=2.408$, $p=0.039$). B6J males and females travel a similar distance for the first 15 minutes in the open field, however, after 15 minutes, the sex differences become apparent with females traveling more than males (Fig 4.2A). Between 15-20 minutes, B6J females travel $1.74\text{m} \pm 0.57\text{m}$ more than B6J males ($F_{1,32}=9.407$, $p=0.004$), and between 25-30 minutes, B6J females travel $1.69\text{m} \pm 0.070\text{m}$ more than B6J males ($F_{1,32}=5.931$, $p=0.021$). Overall, there is a steady decline in TDT over the 30-minute time period for both sexes. For the D2J strain, there was a statistically significant three-way interaction between time, sex, and diet in D2J mice ($F_{5,145}=2.930$, $p=0.015$). Unlike the B6J strain, the D2J strain increased their TDT between 5-10 minutes in the open field, then displayed a steady decline in TDT after 10 minutes (Figure 4.2B). Differences between D2J males and females emerge between 10-15 minutes in the open field, with males traveling more distance overall than females. Specifically, between 10-15 minutes in the open field, D2J males traveled $2.05\text{m} \pm 0.80\text{m}$ more than D2J females ($F_{1,29}=6.493$, $p=0.016$),

and between 20-25 minutes, D2J males traveled $2.06\text{m} \pm 0.81\text{m}$ more than D2J females ($F_{1,29}=6.433$, $p=0.017$).

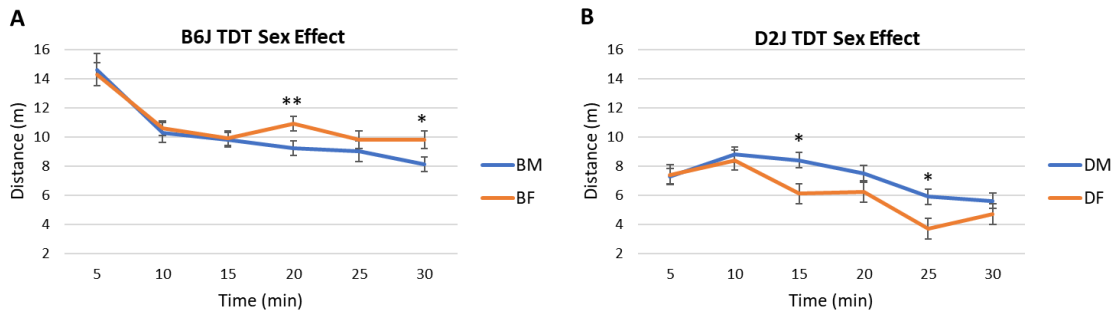


Figure 4.2. Total Distance Traveled Sex Effect with Time. The effect of sex on TDT for B6J mice (A) and D2J mice (B). * $p<0.05$, ** $p<0.01$. Abbreviations: B=B6J, D=D2J, M=male, F=female.

For the between-subjects analysis, there was a main effect of diet for both the B6J strain ($F_{1,32}=42.648$, $p<0.0001$) and the D2J strain ($F_{1,29}=13.700$, $p=0.001$). For B6J males, there was a statistically significant decrease in TDT for mice fed a HFD at each recorded time interval (Figure 4.3A). For B6J female mice fed a HFD, TDT was significantly reduced at all time points except for the first 5 minutes, which was approaching significance (Figure 4.3B). For D2J males, there was an overall reduction in TDT for mice fed a HFD, but the reduction was not as significant compared to B6J males (Figure 4.3C). For D2J females, only the last five minutes in the open field revealed a statistically significant decrease in TDT for mice fed a HFD, although the time period of 10-15 min was approaching significance (Figure 4.3D). Mean TDT data for each treatment group at each time interval are included in Supplementary Table A7.

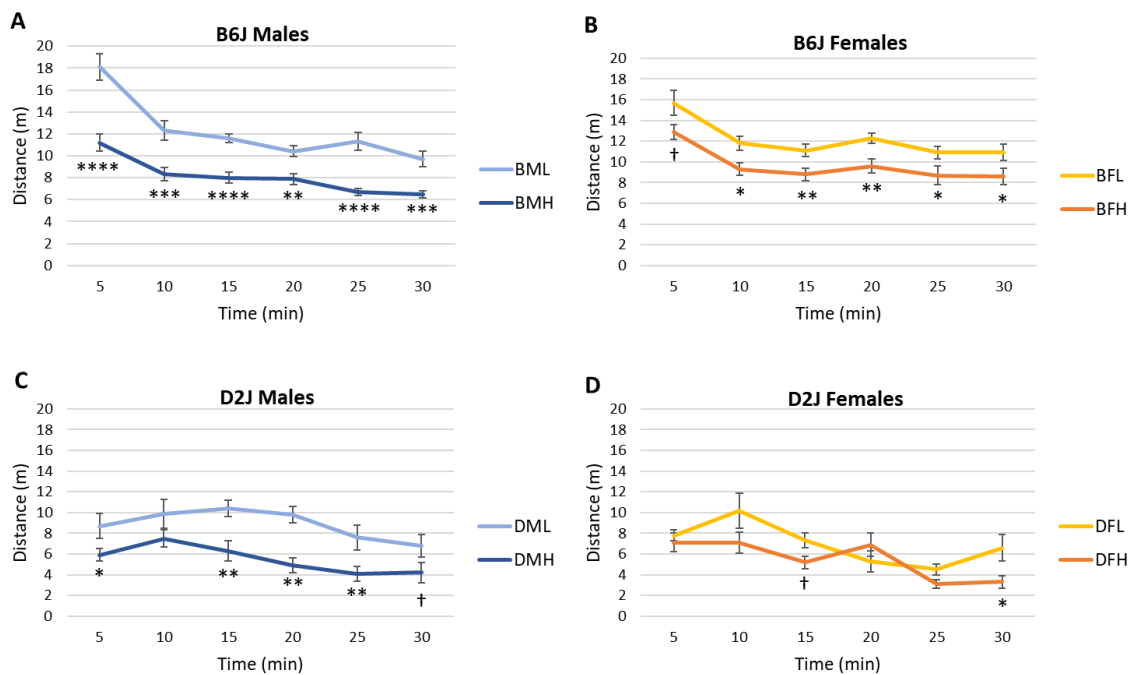


Figure 4.3. Total Distance Traveled Diet Effect with Time. The effect of diet on TDT for B6J male mice (A), B6J female mice (B), D2J male mice (C), and D2J female mice (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, † approaching significance. Abbreviations: B=B6J, D=D2J, M=male, F=female, L=LFD, H=HFD.

B6J and D2J Mice Fed a HFD Have Reduced Velocity in the Open Field

A three-factor mixed ANOVA was used to evaluate the effects of time, diet, and sex on velocity, with diet and sex as the between subject factors and time as the within subject factor. Velocity was analyzed in six 5-minute time intervals for a total of 30 minutes. In the repeated measures within-subject analysis, there was a statistically significant main effect of time on velocity in B6J mice ($F_{5,160}=51.492$, $p < 0.0001$). Two interactions for the B6J strain were approaching significance: time by diet ($F_{5,160}=2.053$, $p=0.074$) and time by sex ($F_{5,160}=1.971$, $p=0.086$). B6J females traveled $5.67 \text{ mm/s} \pm 1.90 \text{ mm/s}$ faster than B6J males between 15-20 minutes in the open field ($F_{1,32}=8.936$,

$p=0.005$) and $5.39 \text{ mm/s} \pm 2.33 \text{ mm/s}$ faster than B6J males between 25-30 minutes ($F_{1,32}=5.374$, $p=0.027$) (Figure 4.4A). For the D2J strain, there was a statistically significant three-way interaction between time, sex, and diet on velocity ($F_{5,140}=3.358$, $p=0.007$). In contrast to the B6J strain, D2J males traveled $6.06 \text{ mm/s} \pm 2.62 \text{ mm/s}$ faster than D2J females between 10-15 minutes in the open field ($F_{1,28}=5.362$, $p=0.028$) and $6.69 \text{ mm/s} \pm 2.83 \text{ mm/s}$ faster than D2J females between 20-25 minutes ($F_{1,28}=5.605$, $p=0.025$) (Figure 4.4B).

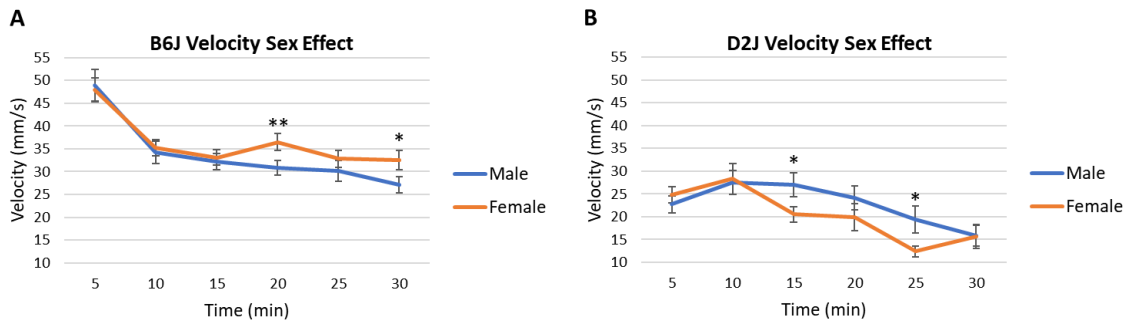


Figure 4.4. Velocity Sex Effect with Time. The effect of sex on velocity for B6J mice (A) and D2J mice (B). * $p<0.05$, ** $p<0.01$.

For the between subject analysis, there was a significant effect of diet for both the B6J strain ($F_{1,32}=42.956$, $p<0.0001$) and the D2J strain ($F_{1,28}=10.674$, $p=0.003$). Additionally, an interaction between diet and sex that was approaching significance for the B6J strain ($F_{1,32}=2.939$, $p=0.096$). B6J males fed a HFD had a substantial reduction in velocity at each time interval in the open field (Figure 4.5A). B6J females fed a HFD also had a reduction in velocity at most time intervals, although not as significant of a change compared to male B6J mice (Figure 4.5B). D2J male mice fed a HFD had reduced

velocities for three time intervals midway through testing (Figure 4.5C). Female D2J mice fed a HFD only had a significantly reduced velocity during the last time interval (25-30 minutes), although reductions in velocity between 5-15 min were approaching significance (Figure 4.5D). Mean velocity data for each treatment group at each time interval are included as Supplementary Table A8.

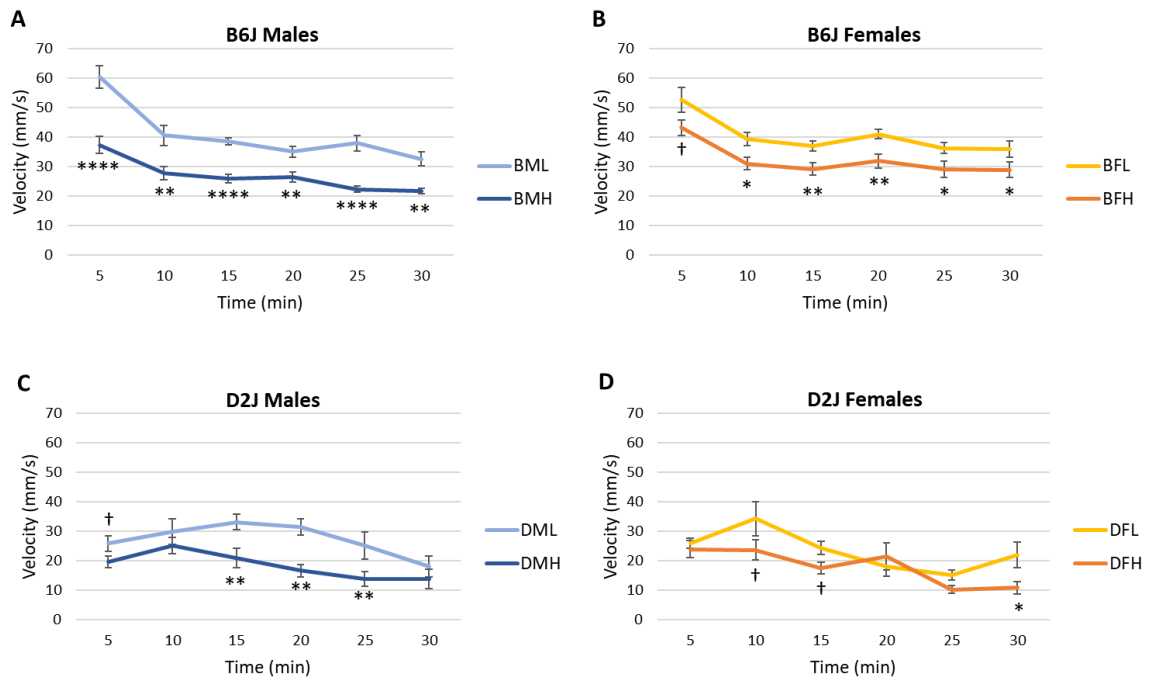


Figure 4.5. Velocity Diet Effect with Time. The effect of diet on velocity for B6J male mice (A), B6J female mice (B), D2J male mice (C), and D2J female mice (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, † approaching significance. Abbreviations: B=B6J, D=D2J, M=male, F=female, L=LFD, H=HFD.

Final Body Weight is Correlated to TDT and Velocity for Females Fed a HFD

Pearson correlation tests were used to assess relationships between final body weight and TDT or velocity. When evaluating all mice combined, there was a moderate negative correlation between body weight and TDT ($R = -0.40$, $p = 0.001$) (Figure 4.6A) and a moderate negative correlation between body weight and velocity ($R = -0.38$, $p = 0.001$) (Figure 4.6B). However, when splitting the data by strain, sex, and diet, the correlations are only significant for females fed a HFD. D2J females fed a HFD had a strong negative correlation between weight and TDT ($R = -0.82$, $p = 0.007$) (Figure 4.6C) and a strong negative correlation between weight and velocity ($R = -0.79$, $p = 0.011$) (Figure 4.6D). A similar trend was approaching significance for B6J females with a strong negative correlation between weight and TDT ($R = -0.66$, $p = 0.054$) (Figure 4.6E) and a strong negative correlation between weight and velocity ($R = -0.66$, $p = 0.052$) (Figure 4.6F).

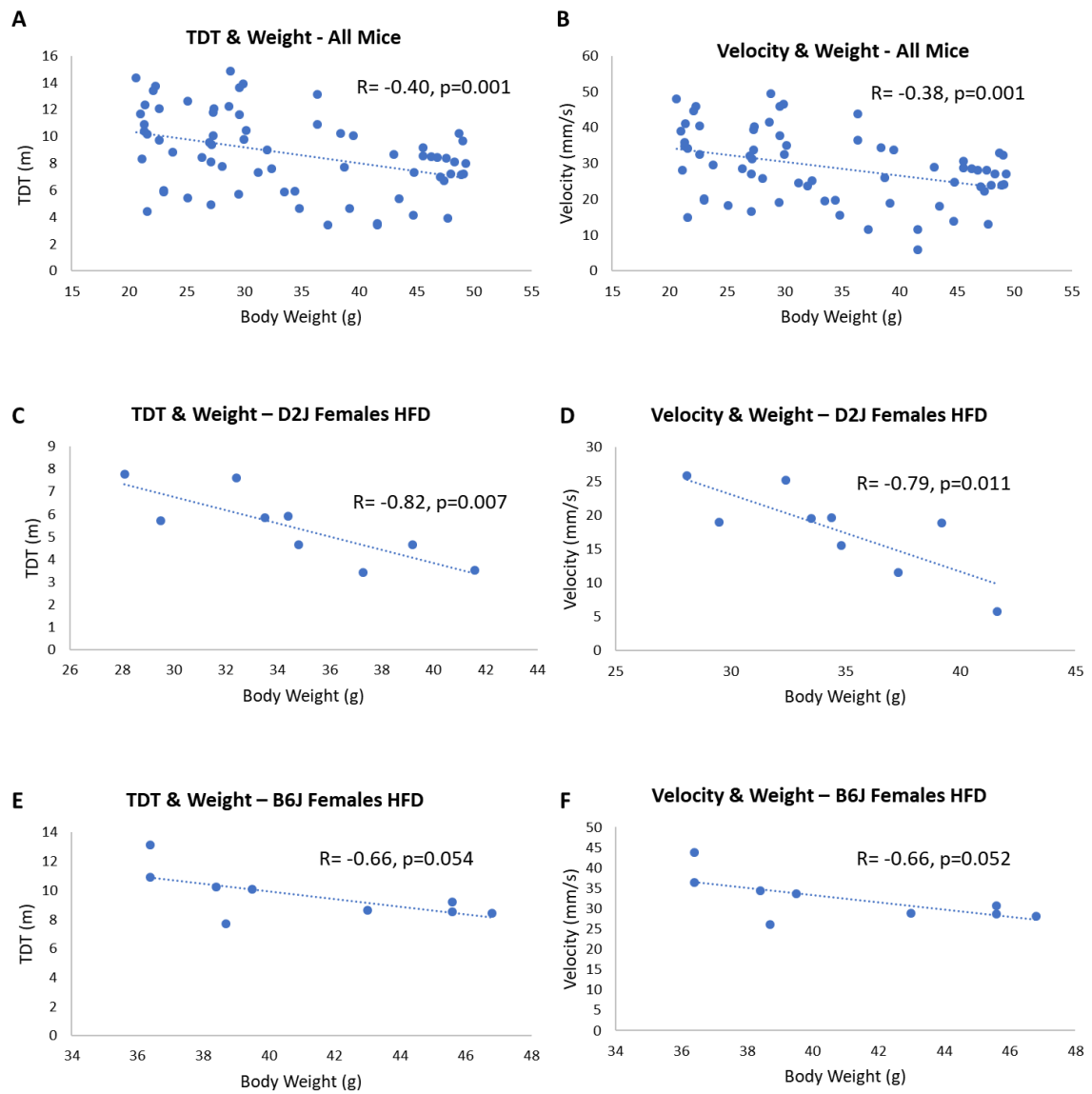


Figure 4.6. Relationships between Weight and Total Distance Traveled or Velocity. Pearson correlations are pictured here for body weight with the following variables: TDT for all mice (A), velocity for all mice (B), TDT for D2J females fed a HFD (C), velocity for D2J females fed a HFD (D), TDT for B6J females fed a HFD (E), and velocity for B6J females fed a HFD (F).

Male B6J Mice Fed a HFD Have a Slower Habituation Rate

The habituation rates in the open field were analyzed separately for each strain due to major differences in locomotion. For the B6J strain, there was a significant main effect of diet on habituation rate. B6J mice fed a HFD had a 35% lower habituation rate compared to B6J mice fed a LFD ($F_{1,32}=4.289$, $p=0.047$). Pairwise comparisons showed that the simple main effect of diet was significant for B6J male mice ($F_{1,32}=5.446$, $p=0.026$) with a 47% reduction in habituation rate for the HFD group (Figure 4.7A). Additionally, there was a main effect of sex on habituation rate for the B6J strain. B6J female mice had a 42% lower habituation rate compared to B6J males ($F_{1,32}=5.928$, $p=0.021$). The simple main effect for sex was significant at the level of LFD ($F_{1,32}=6.713$, $p=0.014$) with B6J females displaying a 53% lower habituation rate compared to males. For the D2J strain, there were no main effects or interactions. Mean habituation rates for each treatment group are provided in Supplementary Table A9.

Male B6J Mice Fed a HFD Show Higher Anxiety-like Behavior Through Fecal Boli

Fecal boli were counted at the end of each open field test as a measure of anxiety level. A three-way ANOVA was used to evaluate differences. Male B6J mice fed a HFD had 37% higher fecal boli compared to B6J mice fed a LFD ($F_{1,63}=5.620$, $p=0.021$) (Figure 4.7B). There was a significant main effect of sex ($F_{1,63}=5.377$, $p=0.024$), with males showing 22% greater fecal boli counts compared to females. This effect of sex was greatest for B6J mice fed a HFD, with a 50% difference between sexes ($F_{1,63}=9.103$,

$p=0.004$). There was no impact of diet or sex on fecal boli for the D2J strain. Mean fecal boli counts for each treatment group are provided in Supplementary Table A9.

DIO Did Not Impact Center Entries in the Open Field

A two-way ANOVA was used to evaluate the impact of sex and diet on the number of center entries in the open field. Center entries are inversely proportional to anxiety-like behavior in rodents. There were no interaction effects on center entries. For B6J mice, the main effect of diet was approaching significance ($F_{1,32}=3.232$, $p=0.082$), as was the main effect of sex ($F_{1,32}=3.759$, $p=0.061$). Center entries for each treatment group are shown in Figure 4.7C. Data for mean center entries for each treatment group are provided in Supplementary Table A9.

Male B6J Mice Fed a HFD Have Significantly Lower Levels of Nestlet Shredding

Nestlet shredding was measured as an assessment of motivation, compulsivity, and general welfare. An example of a shredded nestlet square is provided as Supplementary Figure B6. Since the data within each treatment group was normal but did not pass the test for homogeneity of variance, an unequal variance t test (Welch's test) was used to compare differences in shredding between diet groups. Male B6J mice fed a HFD had 183% less nestlet shredding compared to male B6J mice fed a LFD ($t_{8.041}=3.001$, $p=0.017$) (Figure 4.7D). Female D2J mice fed a HFD had 79% less shredding compared to the LFD group, which was approaching statistical significance ($t_{15}=1.963$, $p=0.068$). Data for mean percent nestlet shredding for each treatment group are provided in Supplementary Table A9.

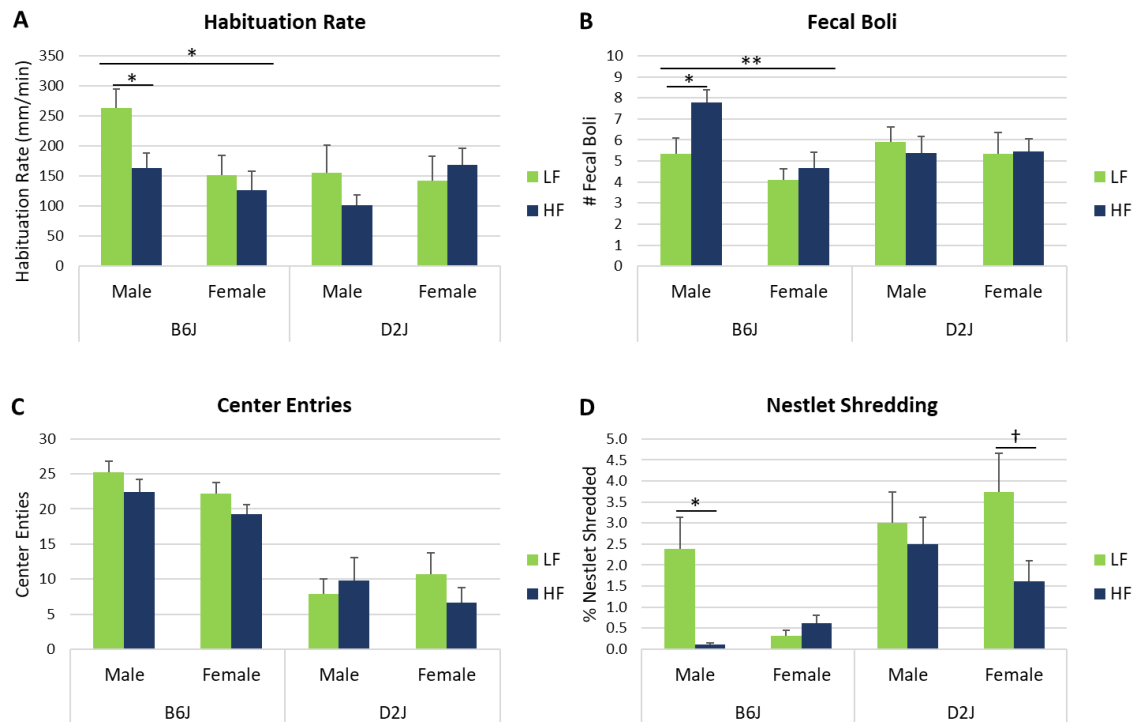


Figure 4.7. DIO Impact on Various Behaviors in B6J and D2J Mice. Behavior testing was assessed using habituation rate (A), fecal boli in the open field (B), center entries into the open field (C), and nestlet shredding (D). Graphs A and B show a sex effect in the B6J strain and a diet effect in male B6J mice for both habituation and fecal boli. Graph C shows trends in center entries with no statistically significant differences. Graph D shows a diet effect for nestlet shredding in B6J males and a diet effect that is approaching significance in D2J females. Data are represented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, †approaching significance. LF=low fat diet, HF=high fat diet.

DIO Did Not Impact Memory in NOR

The NOR test was performed to assess memory in male and female B6J and D2J mice. A two-factor ANOVA was used to evaluate the effects of diet and sex within each strain separately due to heterogeneity of variance between strains. There were no statistically significant main effects or interactions for either strain. All treatment groups except for the D2J females fed a HFD had a positive discrimination index score

(Figure 4.8). Mean discrimination index values with SEM are reported in Supplementary Table A9.

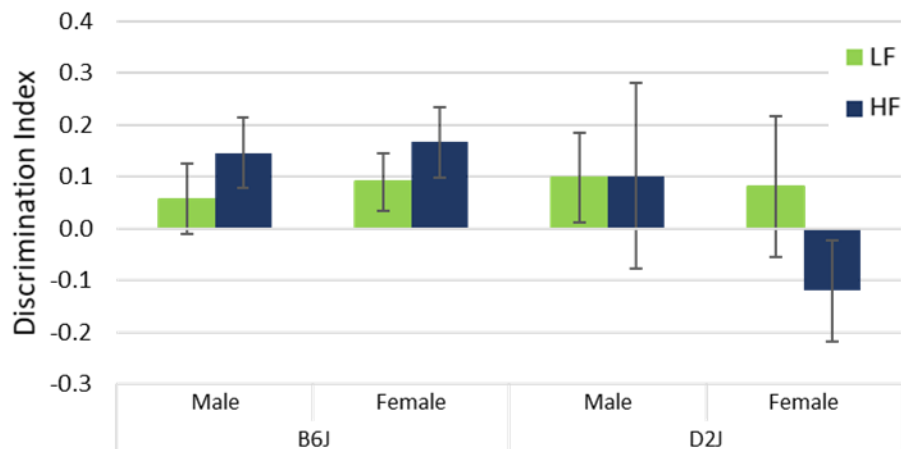


Figure 4.8. Novel Object Recognition in Male and Female B6J and D2J Mice. Positive discrimination index values indicate normal behavior. Data are represented as mean \pm SEM.

Dopamine Release in the Dorsal Striatum is Increased in B6J Females Fed a HFD

Dopamine biology was evaluated in the dorsal striatum, a brain region involved in locomotion and habitual behaviors. There was a statistically significant three-way interaction between diet, sex, and strain on relative dopamine release in the dorsal striatum ($F_{1,40}=5.426$, $p=0.025$). Upon further analysis, there was a statistically significant simple two-way interaction between diet and sex for B6J mice ($F_{1,40}=7.125$, $p=0.011$), with a 24% increase for B6J females and a 10% decrease for B6J males due to HFD. Pairwise comparisons reveal that this 24% increase for female B6J mice was statistically significant ($F_{1,40}=10.142$, $p = 0.003$) but the 10% decrease for male B6J mice was not ($F_{1,40}=0.954$, $p=0.335$) (Figure 4.9A).

Dopamine Reuptake in the Dorsal Striatum is Reduced for D2J Mice Fed a HFD

A three-factor ANOVA was used to evaluate differences between strain, sex, and diet on dopamine transporter Vmax to assess dopamine reuptake. There was a statistically significant two-way interaction between diet and strain on Vmax in the dorsal striatum ($F_{1,40}=4.880$, $p=0.033$). For the D2J strain, mice fed a HFD had a significant 33% reduction in Vmax compared to D2J mice fed a LFD ($F_{1,40}=8.505$, $p=0.006$). However, the B6J strain Vmax differed by only 0.5% ($F_{1,40}=0.096$, $p=0.758$). Pairwise comparisons show that the DIO-induced reduction in Vmax for D2J mice was significant for both males and females, with a 34% reduction for male D2J mice ($F_{1,40}=4.122$, $p=0.049$) and a 32% reduction for female D2J mice ($F_{1,40}=4.431$, $p=0.042$) (Figure 4.9B). Dopamine release and reuptake data in the dorsal and ventral striatum are reported in Supplementary Table A10.

Dopamine Reuptake in the NAc Core is Reduced for Male Mice Fed a HFD

Dopamine biology was also evaluated in the NAc core of the ventral striatum, a brain region involved in motivation, anxiety, and addictive behaviors. Dopamine release in the NAc was not significantly impacted by DIO, however there was a main effect of strain ($F_{1,44}=4.891$, $p=0.032$), with D2J mice showing an 11% greater dopamine release compared to B6J mice. Dopamine release in the NAc core for each treatment group is shown in Figure 4.9C. There was a significant diet by sex interaction on Vmax ($F_{1,44}=4.645$, $p=0.037$), with males showing a significant reduction in Vmax ($F_{1,44}=9.252$, $p=0.004$) compared to females ($F_{1,44}=0.059$, $p=0.810$) due to HFD. Pairwise comparisons

show that this obesity-induced decrease is statistically significant in D2J males with a 49% reduction in Vmax ($F_{1,44}=6.932$, $p=0.012$) and approaching significance for B6J males with a 56% reduction ($F_{1,44}=3.300$, $p=0.076$) (Figure 4.9D). Furthermore, there was a main effect of strain ($F_{1,44}=7.209$, $p=0.010$), with D2Js exhibiting a 26% higher Vmax compared to the B6J strain.

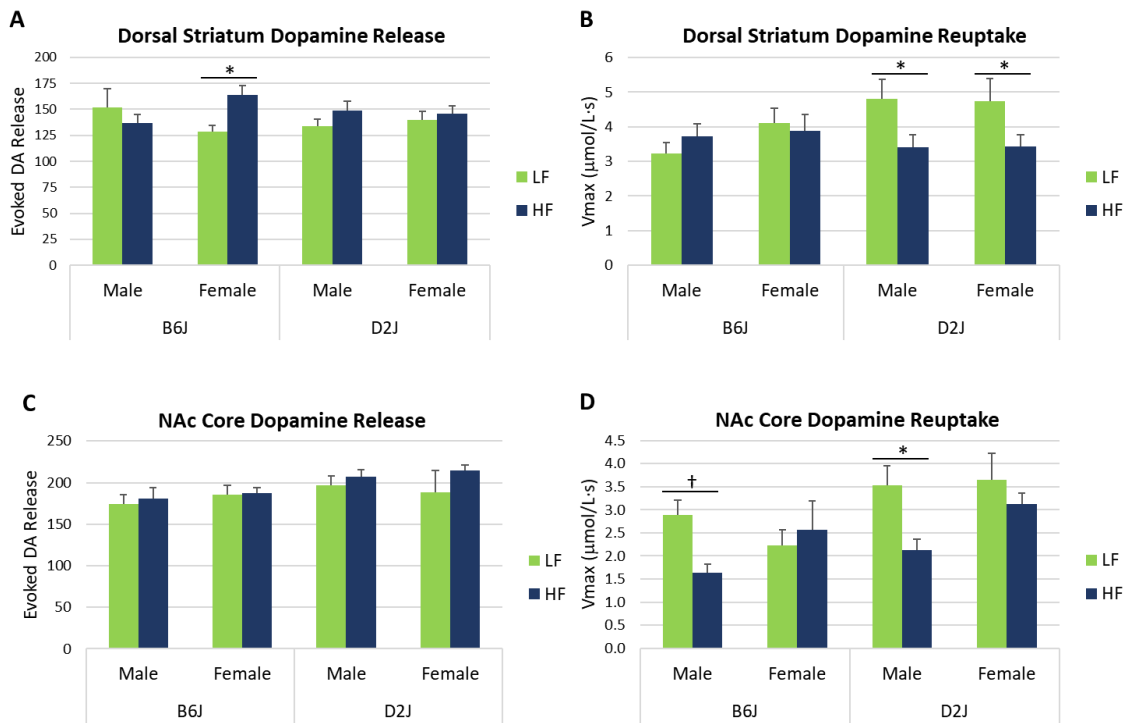


Figure 4.9. Dopamine Release and Reuptake in the Striatum. This figure shows the impact of diet on dopamine release in the dorsal striatum (A), DAT-mediated dopamine reuptake (Vmax) in the dorsal striatum (B), dopamine release in the NAc core (C), and DAT-mediated dopamine reuptake (Vmax) in the NAc core (D). Data are represented as mean \pm SEM. * $p<0.05$, †approaching significance. DA = dopamine, DAT = dopamine transporter, NAc = nucleus accumbens, Vmax = maximal rate of dopamine reuptake.

In the Striatum, DRD2 Gene Expression was Upregulated in B6J Males Due to DIO and TH Gene Expression was Significantly Higher in the B6J Strain Compared to the D2J Strain

DRD2 and TH mRNA expression were measured in the striatum. Male B6J mice fed a HFD showed a 2.40 ± 0.70 fold increase in gene expression compared to B6J males fed a LFD ($F_{1,24}=4.241$, $p=0.049$) (Figure 4.10A). DIO did not have an impact on mRNA expression of DRD2 in the other treatment groups. Furthermore, DIO had no impact on tyrosine hydroxylase mRNA expression in the striatum (Figure 4.10A). However, there was a significant main effect of strain ($F_{1,30}=173.528$, $p<0.0001$), with B6J mice expressing 8.08 ± 0.76 fold more tyrosine hydroxylase compared to D2J mice (4.10B). Ct values for DRD2 and TH gene expression in the striatum are provided in Supplementary Table A3.

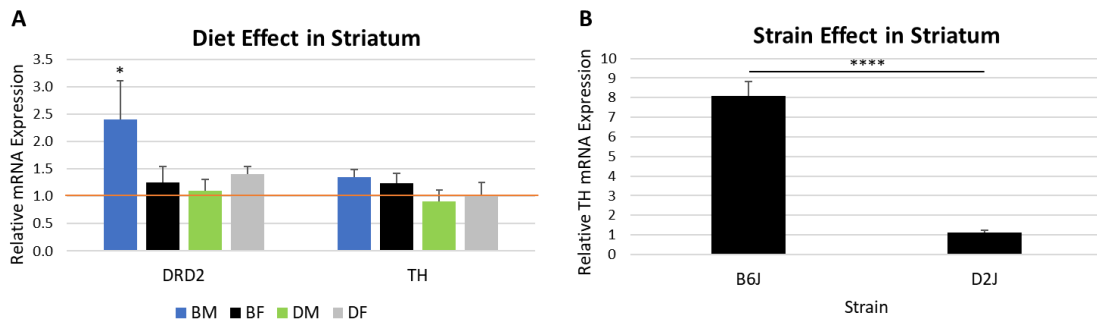


Figure 4.10. Striatum DRD2 and TH Gene Expression. The effect of diet on DRD2 and TH mRNA gene expression (A) and the effect of strain on TH mRNA gene expression (B) in the striatum as fold change compared to a reference. Fold change compared to each control was determined using the comparative Ct method. For the diet effect, the LFD mRNA expression was established as the control (LFD reference = 1 as indicated by the horizontal bar). For the strain effect, the D2J strain was set as the control. Data are represented as mean \pm SEM. * $p<0.05$, **** $p<0.0001$. DRD2 = dopamine transporter D2, TH = tyrosine hydroxylase, BM=B6J males, BF=B6J females, DM=D2J males, DF=D2J females.

In the Hippocampus, Female D2J Mice Express Significantly More BDNF Compared to D2J Males

In the hippocampus, the mRNA expression of BDNF was evaluated. There was no effect of DIO on BDNF expression in this brain region. However, D2J females expressed 2.54 ± 0.19 fold more BDNF compared to D2J males ($F_{1,30}=32.677$, $p<0.0001$) (Figure 4.11). Ct values for BDNF gene expression in the hippocampus are provided in Supplementary Table A4.

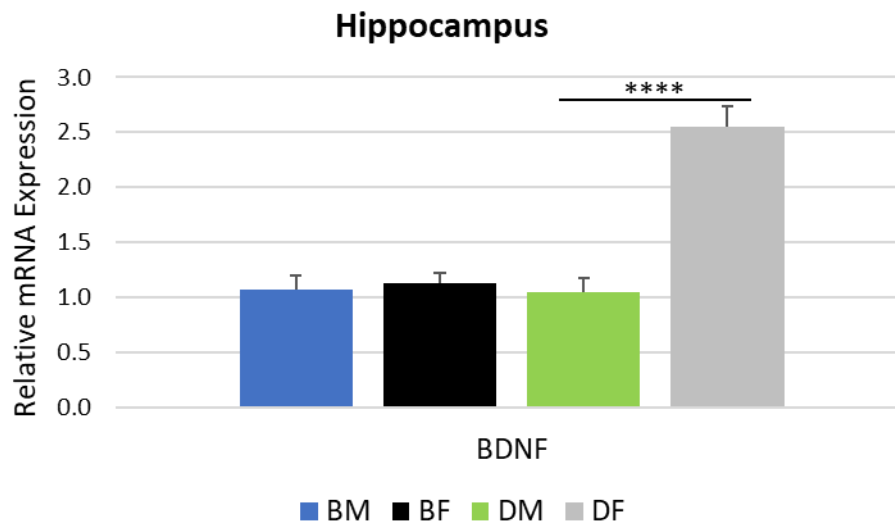


Figure 4.11. Influence of Sex on BDNF Gene Expression in the Hippocampus. Fold change comparisons by sex are shown for BDNF in the hippocampus. Male mRNA expression for each strain was established as the control. Fold change in female mRNA expression compared to each control was determined using the comparative Ct method. Data are represented as mean \pm SEM. **** $p<0.0001$. BDNF = brain-derived neurotrophic factor, BM=B6J males, BF=B6J females, DM=D2J males, DF=D2J females.

In the Olfactory Bulb, DIO Caused an Upregulation of DRD2 and TH Gene Expression in Male D2J Mice and Induced BDNF Gene Expression in Female B6J Mice

In the olfactory bulb, mRNA expression was evaluated for DRD2, TH, and BDNF. There was a statistically significant induction of DRD2 in D2J male mice fed a HFD by 3.52 ± 1.03 fold ($F_{1,24}=5.879$, $p=0.025$) and a downregulation of DRD2 in B6J male mice fed a HFD by 0.48 ± 0.16 relative to control (approximately 2-fold decrease) was approaching significance ($F_{1,24}=3.868$, $p=0.064$) (Figure 4.12). For TH gene expression, there was a diet by strain interaction in the olfactory bulb ($F_{1,16}=7.213$, $p=0.016$). Pairwise comparisons showed that the diet effect was significant only for D2J males, with a 3.78 ± 1.26 fold upregulation due to HFD ($F_{1,16}=6.444$, $p=0.022$) (Figure 4.12). For BDNF expression, there was a diet by sex interaction ($F_{1,24}=4.378$, $p=0.047$). Female mice fed a HFD had 1.87 ± 0.39 fold higher BDNF expression compared to female mice fed a LFD ($F_{1,30}=7.811$, $p=0.10$), but there was no significant difference in expression for males. Pairwise comparisons revealed that B6J females fed a HFD were impacted the greatest, with an upregulation of BDNF by 2.08 ± 0.39 fold (Figure 4.12). Ct values for DRD2, TH, and BDNF gene expression in the olfactory bulb are provided in Supplementary Table A5.

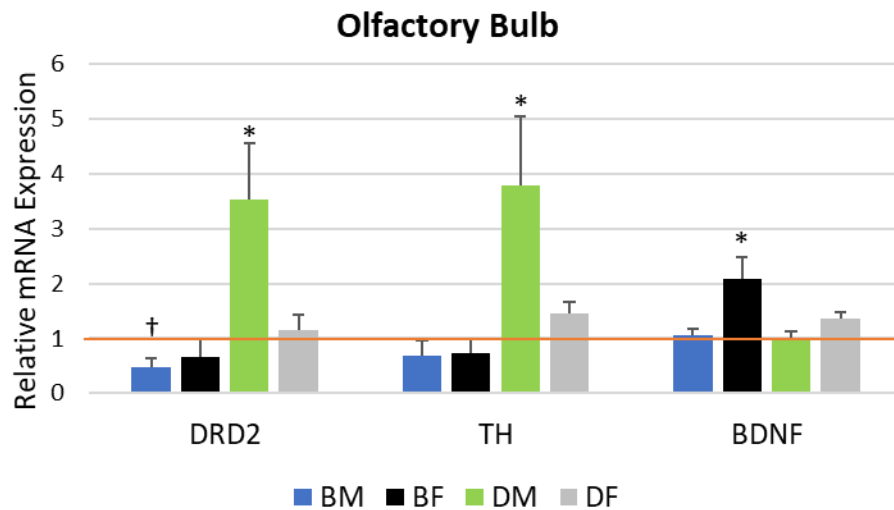


Figure 4.12. DIO Impact on Olfactory Bulb DRD2, TH, and BDNF Gene Expression. Fold change comparisons by diet for these genes are shown for the olfactory bulb. The LFD mRNA expression was established as the control (LFD reference = 1 as indicated by the horizontal bar). Fold change compared to each control was determined using the comparative Ct method. Data are represented as mean \pm SEM. * $p < 0.05$, †approaching significance. DRD2 = dopamine transporter D2, TH = tyrosine hydroxylase, BDNF = brain-derived neurotrophic factor, BM=B6J males, BF=B6J females, DM=D2J males, DF=D2J females.

Discussion

The objective of our study was to determine sex and strain differences on behavior, dopamine biology, and gene expression in normal and obese male and female B6J and D2J mice. Our main findings were as follows: 1) mice with DIO had reduced motor activity, 2) B6J male mice with DIO displayed reduced habituation, decreased motivation, and increased anxiety-like behavior, 3) dopamine clearance in the dorsal striatum was reduced in D2J mice with DIO, 4) dopamine clearance in the NAc core was reduced in male mice with DIO, 5) DRD2 was upregulated in male B6J mice with DIO in the striatum, 6) DRD2 and TH were upregulated in male D2J mice with DIO in the olfactory bulb. Our hypothesis that DIO would have a greater impact on the B6J strain

compared to the D2J strain was confirmed for several behavior tests, however, the dopamine biology dysregulation and gene expression alterations impacted both strains. Likewise, our hypothesis that DIO would impact males more than females was validated for behavior testing, dopamine uptake in the NAc core, and gene expression of DRD2 and TH.

Weight Gain

In the current study, all mice fed a lard-based HFD with 60% kcal from fat gained a significant amount of weight compared to mice fed a LFD (Figure 4.1). This weight gain is consistent with previous reports using B6J and D2J strains for DIO investigations (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992) and previous DIO studies from our lab using male B6J mice (Han et al., 2019; Liu et al., 2016). The diet treatment was initiated at a young age of approximately three weeks old and continued for 16 weeks. Based on the significant weight gain that we observed and measured in both male and female B6J and D2J mice, we can conclude that this diet regimen provided a successful model for the study of DIO on brain and behavior dysregulation.

DIO and Locomotion

Obesity has been associated with reduced physical activity in both humans (Forhan and Gill, 2013; Trivedi et al., 2015) and rodents (Almeida-Suhett et al., 2017; Krishna et al., 2016). In our study, we measured TDT and velocity in five-minute time bins over a period of 30 minutes in the open field for each treatment group. Overall, TDT and velocity decreased over time for all treatment groups as expected (Figures 4.3

and 4.5). We found a significant time by sex interaction on TDT for B6J mice, with females traveling more than males during the last 15 minutes of the test (Figure 4.2A). The D2J strain displayed different locomotor behavior compared to the B6J strain. D2J mice travel minimally during the first five minutes, then proceed to travel more between 5-10 minutes followed by a steady decline in TDT over the remaining 20 minutes. Furthermore, there was a significant three-way interaction between time, sex and diet on TDT for D2J mice. Unlike the B6J strain, male D2J mice traveled more than female D2J mice, most notably between 10-15 minutes and 20-25 minutes in the open field (Figure 4.2B). For both strains, there was a main effect of diet on reducing TDT, however this effect was more significant for the B6J males and females (Figures 4.3A and B) compared to the D2J males and females (Figures 4.3C and 4.3D). The impact of DIO on reducing TDT in male B6J mice in our study (Figure 4.3A) was consistent with other DIO studies using male B6J or B6 mice (Almeida-Suhett et al., 2017; Gelineau et al., 2017; Tsai et al., 2018; Wu et al., 2018). TDT was also significantly reduced in our female B6J mice fed a HFD at most time intervals (Figure 4.3B). Additionally, there was a negative correlation between body weight and TDT that was significant for female D2J mice (Figure 4.6C) and trending toward significance for female B6J mice (Figure 4.6E). In other studies using both male and female B6 or B6J mice fed a HFD (60% kcal fat), there was no effect of DIO on TDT in the female mice (Bridgewater et al., 2017; Gelineau et al., 2017). However, these studies initiated their HFD at approximately six weeks of age (compared to ours at three weeks of age) with a diet duration of 10-12 weeks

(compared to ours for a 16-week duration). Furthermore, a study using female B6 mice fed a 60% HFD starting at 6-7 weeks old found that mice with DIO showed reduced locomotion after approximately 32 weeks of HFD feeding, but not at 21 weeks of HFD feeding (Krishna et al., 2016). Again, this study initiated the HFD at a later age, and used a substrain of B6 mice with a different genetic background compared to our B6J mice. Early initiation of the HFD to produce the DIO state could explain the overall reduction in TDT that we see in both sexes and both strains in our study, indicating a more serious health threat to physical activity when a HFD begins early in life.

DIO and Habituation

Habituation in rodents represents a natural reduction in locomotor activity after an initial period of exploration in a novel environment. This learning behavior allows mice to discriminate between novel and normal stimuli. Abnormal habituation behavior has been associated with lesions in the hippocampus and disruptions in dopamine biology (Leussis and Bolivar, 2006). In our study, B6J and D2J habituation data were analyzed separately due to major differences in ambulation and heterogeneity of variance. Habituation was determined using two methods: first by visual inspection of TDT over time (Figure 4.3) and second by regressing distance traveled over time and using the regression coefficients (slopes) to determine habituation rates (Figure 4.7A). Over the 30-minute testing period, both strains and sexes showed an overall reduction in locomotion as assessed by total distance traveled (Figure 4.3), indicating normal habituation trends. This is consistent with other studies using male B6J and D2J mice

(Anisman et al., 1976; Cabib et al., 1990; Kafkafi et al., 2003) and a combination of male and female B6J and D2J mice (Koyner et al., 2000). One study using a shorter open field session found D2J activity to increase over time, while B6J activity decreased (Logue et al., 1997). This is also consistent with our data, which show an increase in activity for D2J mice over the first 5-10 minutes. During the open field test, we observed that the D2J strain often remained still for several minutes before starting to explore, explaining the rise in activity during the first 10 minutes. Remaining still is a reflection of anxiety when first introduced to the open field environment (Gould et al., 2009). Nevertheless, all mice in our study habituated to the open field when accounting for the full 30-minute test session. Specific habituation rates for each treatment group were calculated based on the overall 30-minute open field test (Figure 4.7A). Our data reveal a main effect of diet in B6J mice, with mice fed a HFD displaying a 35% reduction in habituation rate. Furthermore, pairwise comparisons showed that the diet effect had the biggest impact on male B6J mice, with a 47% reduction in habituation rate due to DIO. This significant result in male B6J mice indicates potential disruptions in the hippocampus, as this brain region is associated with the habituation process (Bolivar, 2009; Leussis and Bolivar, 2006). In a previous study from our lab using the same mice, we reported a significant upregulation in alpha synuclein (7-fold) and amyloid precursor protein (APP) (10-fold) mRNA transcripts in the hippocampus of B6J mice fed a HFD. Although we did not measure protein expression in that study, it is possible that the upregulation of alpha synuclein and APP could potentiate neurodegeneration in this brain region, thus

impacting habituation. In addition to the diet effect on habituation in our current study, we also found a significant sex effect in B6J mice, with females habituating at a 42% reduced rate compared to males. There were no significant diet or sex effects within the D2J strain, indicating that the D2J strain is more resistant to sex and diet effects compared to the B6J strain when comparing the rate of habituation.

DIO and Anxiety-like Behavior

There is evidence in humans (Baker et al., 2017; Gariepy et al., 2010; Strine et al., 2008) and in rodents (Almeida-Suhett et al., 2017; Krishna et al., 2016) that suggests a relationship between obesity and anxiety. In the current study, we assessed anxiety in the open field arena by measuring avoidance of center entries and fecal boli quantity. Evading the center area and fecal boli are common behavioral assays for determining anxiety-like behavior in mice that are introduced to a novel environment (Seibenhener and Wooten, 2015). Our data revealed a significant 37% increase in fecal boli produced by male B6J mice fed a HFD compared to mice fed a LFD (Figure 4.7B). There was also a significant sex effect in the B6J strain, with males producing 50% more fecal boli compared to females (Figure 4.7B). The impact of DIO on the B6J strain is consistent with another study that reported an increase in fecal boli for male and female SM/J mice fed a HFD, however, this study showed no sex effect with the SM/J strain (Keleher et al., 2018). In our study, there was no sex or diet effect on fecal boli in the D2J strain, which highlights the influence of genetics on the impact of DIO on specific types of behavior. Evaluation of center entries in the present study showed only a slight

decrease for mice fed a HFD in most treatment groups. It is interesting to note the disparities in the literature regarding center entries and percent center time for the assessment of DIO impact on anxiety-like behavior in B6J or B6 mice. For example, one study in male B6J mice found a negative correlation between weight gain and center entries (Almeida-Suhett et al., 2017), while others using B6 (Tsai et al., 2018) or B6J (Zilkha et al., 2017) mice found no effect of DIO on center entries. In a study with female B6 mice, there was no impact of DIO on center entries after 12 and 21 weeks of HFD feeding, but DIO mice had reduced center entries after 32 weeks of HFD feeding (Krishna et al., 2016, 2015). There are several factors that may explain these different results, such as the size of the open field, the age at which HFD is initiated, diet duration, and the use of different substrains of B6 mice. Although it is recommended by some protocols to test for anxiety within the first 5-10 minutes of the open field test (Gould et al., 2009; Seibenhener and Wooten, 2015), a wide variety of time frames are used in DIO studies. In our study, we used the first five-minute time bin (0-5min) for the B6J strain and the second five-minute (5-10) for the D2J strain in order to capture the most active time for each strain, as shown in the TDT and velocity plots (Figures 4.3 and 4.5). Considering the discrepancies in center time or center entry results in DIO studies listed here, it is possible that other behavioral assays for anxiety in rodents may be more accurate predictors of anxiety in obese mice, such as the elevated plus maze, which has been used previously in DIO studies in mice and rats (Agrimi et al., 2019; Bax et al., 2019).

While there is extensive published research on sex differences regarding associations between obesity and depression, less is known about sex factors involved in relationships between obesity and anxiety (Tronieri et al., 2017). Some studies show a more significant relationship between overweight or obesity and anxiety in females compared to males (Anderson et al., 2006; Barry et al., 2008; DeJesus et al., 2016; Hofmann et al., 2015; Svenningsson et al., 2012). However, there are other reports that suggest a higher incidence of anxiety in obese males compared to obese females (Bjerkeset et al., 2007; Bridgewater et al., 2017; Tronieri et al., 2017). Our fecal boli data show that only male B6J mice fed a HFD displayed higher anxiety-like behavior. These results indicate that sex is an influential factor regarding the impact of DIO on the development of anxiety.

DIO Effect on Motivation and Welfare

The nestlet shredding test has been used to evaluate a variety of behaviors, including obsessive-compulsive behavior (Angoa-Pérez et al., 2013), motivation or apathy (Nichols et al., 2016), and general welfare (Gaskill et al., 2013). It has also been used to assess the efficacy of anxiolytics (Li et al., 2006). Nestlet shredding for the purpose of nest building, warmth, or protection is a natural, spontaneous behavior in laboratory mice of both sexes (Gaskill et al., 2013; Jirkof et al., 2013; Nichols et al., 2016). Excessive shredding indicates repetitive, compulsive behaviors (Angoa-Pérez et al., 2013), while latency to shred can be a sign of apathy, depression, or poor health associated with disease progression (Jirkof, 2014; Nichols et al., 2016). In the present

study, we reveal that DIO caused a significant reduction in shredding behavior for male B6J mice (Figure 4.7D). This latency to shred could indicate a lack of motivation caused by chronic exposure to a HFD, or could be a sign of declining nervous system health. Impaired nestlet shredding has been associated with hippocampal dysfunction and neurodegenerative disease, specifically with mouse models of Alzheimer's disease (Jirkof, 2014). Furthermore, mice with hippocampal lesions have performed poorly in nest building tasks and show a similar latency in shredding (Deacon and Rawlins, 2005). Although we did not see any differences in BDNF mRNA expression in the hippocampus in the present study, we did see a significant upregulation of alpha synuclein and APP in the hippocampi of these mice in a companion study. As mentioned above with habituation deficits in male B6J mice, this upregulation of alpha synuclein and APP may be a sign of neurodegeneration in the hippocampus. Interestingly, a recent study in mice exposed to lead (Pb) in drinking water found that the Pb-exposed mice presented with latency to shred (Chang et al., 2014), providing more evidence that the nestlet shredding test is a potential indicator of hippocampal dysfunction. Based on the data presented here and in our previous work with DIO mice, we propose that DIO generated by a HFD acts as an environmental stress that can increase the risk for neurodegeneration and negative behavior transformations.

DIO and Memory

There is evidence in humans that a diet high in saturated fat can lead to memory impairment and an increased risk for Alzheimer's disease (Eskelinen et al., 2008;

Pasinetti and Eberstein, 2008). In rodents, there are mixed results regarding the effect of DIO or HFD on memory. For example, memory was compromised in young male B6J mice fed a HFD (60% kcal fat) for a short duration of one week, with the diet treatment initiated at three weeks old (Kaczmarczyk et al., 2013). A similar result was reported for middle-aged (11 months old) male B6J mice fed a HFD (60% kcal fat) for four months (Carey et al., 2014). In contrast, male B6J mice fed a moderately HFD (32% kcal fat) at 11 weeks old for 6.5 months showed no difference in short- or long-term memory (Tucker et al., 2012). Female B6 mice at age 6-7 weeks old fed a 60% HFD for 12, 22, and 36 weeks also showed that diet had no impact on memory (Krishna et al., 2016, 2015). Under normal diet conditions, there are natural strain differences in memory which show that the B6J strain has superior memory compared to the D2J strain (Lenselink et al., 2015). Our results show that DIO did not have a statistically significant impact on memory as assessed by the novel object recognition test. It is interesting, however, that the discrimination index for female D2J mice fed a HFD was negative, indicating a possible disturbance of normal behavior due to DIO.

Dopamine Release and Reuptake in the Dorsal Striatum

Fast scan cyclic voltammetry was used to measure real-time dopamine release and reuptake by dopamine transporter (DAT) in the striatum. We found that dopamine release in the dorsal striatum was significantly increased by 24% in female B6J mice fed a HFD (Figure 4.9A). Additionally, our data reveal a diet by strain interaction for dopamine reuptake in the dorsal striatum, specifically both male and female D2J mice

fed the HFD showed a decreased rate of dopamine reuptake whereas the B6J were unaffected (Figure 4.9B). In another study with male B6J mice fed a high fat/high sugar Western style diet for 16 weeks, dopamine release in the dorsal striatum was increased and dopamine clearance was decreased in mice fed the Western style diet (Fritz et al., 2018). Although our results were consistent with this study in the direction of change for dopamine release and reuptake, we did not observe these changes in B6J males. DIO studies using male and female rats have consistently reported decreases in dopamine clearance in this subregion of the striatum (Geiger et al., 2009; Morris et al., 2011; Patel et al., 2019), which is similar to what we discovered for the D2J strain. These same rat studies also report decreases in dopamine release; however, we found an increase in release for B6J females, and no change for the other treatment groups. It is possible that the dopamine response is different for mice compared to rats in the dorsal striatum, although research in mice on this topic, especially in females, is currently limited.

Dopamine Release and Reuptake in the NAc Core of the Ventral Striatum

Dopamine release and reuptake were also assessed in the NAc core. In this brain region, we found no impact of DIO on dopamine release in either strain or sex. There was, however, a diet by sex interaction for Vmax, with male mice fed a HFD exhibiting a decreased rate of dopamine clearance compared to female mice. Although the magnitude of reduction was greater in the B6J males (56% less) compared to the D2J males (49% less), the D2J male result was statistically significant, while the B6J male

result was trending toward significance. This decrease in NAc Vmax in male mice is consistent with several other reports. For example, in three separate studies using male B6J mice fed a HFD for six weeks, the use of fast scan cyclic voltammetry revealed a significant decrease in dopamine reuptake in the NAc core (Barnes et al., 2020; Fordahl et al., 2016; Fordahl and Jones, 2017). In the present study, it is interesting that DIO only had an impact on dopamine clearance in males from each strain. It is possible that estrogen has a neuroprotective effect on dopamine biology (Dluzen and McDermott, 2000), specifically in the NAc core of females (Thompson, 1999), which would explain the diet by sex interaction. Our results that show no change in dopamine release in the NAc core are consistent with another study in B6J mice fed a 60% HFD for six weeks (Fordahl et al., 2016), however, other reports in mice and rats fed a HFD have found a decrease in dopamine release in this region (Barnes et al., 2020; Geiger et al., 2009; Patel et al., 2019). It has been reported that DAT may regulate diurnal oscillations of dopamine release in mice and rats (Ferris et al., 2014) and that ovarian hormones in rats can impact circadian variation of dopamine release in the striatum (Becker et al., 1984), which could explain the discrepancies in dopamine release that have been reported in the striatum of rodents. Nevertheless, we show here that DIO has an impact on dopamine biology in a strain- and sex-dependent manner which should be further investigated.

Effect of Diet, Sex, and Strain on DRD2 mRNA Expression

DRD2 has an important role in dopamine biology, facilitating the actions of dopamine that control both movement and reward-seeking (Gallo, 2019). In the current study, we discovered dysregulations in DRD2 mRNA gene expression in the striatum and olfactory bulb due to DIO. For this evaluation, the striatum was not divided into dorsal and ventral subregions, but included the entire brain region. We found a significant upregulation of DRD2 by 2.4-fold in the striatum of B6J males (Figure 4.10A). In the olfactory bulb, we found a significant 3.5-fold upregulation in DRD2 mRNA in male D2J mice, and a 2-fold downregulation in male B6J that was approaching significance (Figure 4.12). DIO did not impact the gene expression in females of either strain.

There are conflicting reports regarding DRD2 expression dysregulation due to DIO, with some reporting downregulation, some upregulation, and a few reporting no change. For example, an imaging study in men and women discovered that striatal DRD2 receptor availability was reduced in obese individuals compared to controls, and that BMI was negatively correlated with DRD2 concentration (Wang et al., 2001). In corroboration using similar test methods, striatal DRD2 receptor availability was reduced in obese women compared to controls (van de Giessen et al., 2014). However, another imaging study in obese women found no change in DRD2 receptor availability when examining several brain regions, including the striatum, and found no correlation between DRD2 and BMI (Karlsson et al., 2015). In a study using male Sprague-Dawley rats fed a HFD for eight weeks, there was a 42% decrease in DRD2 density in the dorsal

striatum (Narayanaswami et al., 2013). Furthermore, when male and female offspring of female B6J mice bred with D2J males were fed a HFD for 12 weeks, both males and females showed a downregulation of DRD2 mRNA in the NAc core (Carlin et al., 2013). In contrast to these studies demonstrating a downregulation or no change in DRD2 expression, several labs have reported an upregulation in DRD2 due to DIO. In a study using male B6 mice fed a HFD for 20 weeks, there was an induction of DRD2 mRNA in the NAc core for mice fed a HFD, and a positive correlation between final body weight and DRD2 gene expression in this brain region (Huang et al., 2005b). Another study using male B6 mice fed a HFD for 12 weeks showed that mice fed a HFD had increased DRD2 protein expression in the NAc (Sharma and Fulton, 2013). Furthermore, when male B6 mice were fed a HFD for only 20 days, the DRD2 binding density was increased in the dorsal and ventral striatum (South and Huang, 2008). Collectively, these studies provide evidence that DRD2 dysregulation is involved as either a cause (in humans) or consequence of DIO, however the direction of DRD2 alteration and pathophysiological mechanisms are poorly understood. The DRD2 upregulation we discovered in male B6J mice in the striatum is consistent with reports in B6 mice described here. Although we did not measure protein or binding density, our mRNA expression results are a unique and valuable contribution for understanding sex and strain differences in the study of DIO impact on neurobiology. To the best of our knowledge, there are no prior published results regarding the impact of DIO on DRD2 expression in the olfactory bulb. As with the striatum, we found that males were susceptible to mRNA dysregulation in the

olfactory bulb, but not females, possibly due to the protective effects of estrogen. The opposite trends in mRNA dysregulation in the olfactory bulb for the B6J and D2J strains also highlight the important influence of genetics on the brain's response to a state of DIO.

Effect of Diet, Sex, and Strain on TH mRNA Expression

TH is a rate limiting enzyme for the synthesis of dopamine, an important neurotransmitter involved in attention, memory, and cognition (Daubner et al., 2011). Furthermore, TH is required for proper motor function (Jang et al., 2017). In the present study, we found a significant 3.8-fold upregulation of TH mRNA in the olfactory bulb for male D2J mice (Figure 4.12). In the striatum, there was no impact of DIO on TH gene expression (Figure 4.10A). However, we found a significant 8-fold increase in TH mRNA expression in the B6J strain compared to the D2J strain (Figure 4.10B). Previous rodent studies have reported an upregulation in TH mRNA expression due to DIO in other brain regions. For example, in a study using male B6 mice fed a HFD for 20 weeks, there was an upregulation of TH mRNA in the midbrain for mice fed a HFD, and a positive correlation between final body weight and TH gene expression in this brain region (Huang et al., 2005a). A study using female C57BL6/129SVJ mice fed a HFD for 12 weeks discovered an induction of TH mRNA expression in the hypothalamus using microarray analysis and real time polymerase chain reaction techniques (Lee et al., 2010). In contrast, other human and rodent studies have found a downregulation of TH expression due to DIO in various brain regions. In a study using postmortem brain tissue

from male and female Caucasians and African Americans, TH mRNA was downregulated in the substantia nigra in obese tissue, but not overweight or control tissue (Wu et al., 2017). A study using male B6 mice fed a HFD for 13 weeks found that TH protein expression was downregulated in the midbrain and striatum for mice fed a HFD (Jang et al., 2017). Furthermore, B6J male mice fed a HFD for just 6 weeks showed a decrease in TH protein expression in the striatum and midbrain, along with increased anxiety-like behavior in mice fed a HFD (Sharma et al., 2013). The induction of TH mRNA that we found in the olfactory bulb of male D2J mice fed a HFD for 16 weeks indicates that the impact of DIO in this brain region is influenced by sex and strain. Moreover, we reveal a substantial 8-fold strain effect in the striatum for this gene, emphasizing the influence of genetics on the expression of genes that are related to dopamine biology. While we do not fully understand the neurophysiological mechanisms behind these sex and strain influences in TH gene expression, our preliminary findings provide a foundation for future work involving the impact of DIO on gene expression dysregulation in the brain.

Effect of Diet, Sex, and Strain on BDNF mRNA Expression

Brain-derived neurotrophic factor (BDNF) is a protein and growth factor involved in neuronal survival and brain plasticity (Bathina and Das, 2015; Miranda et al., 2019). The role of this neurotrophin in brain plasticity is correlated with learning, memory, and cognition in humans and rodents (Miranda et al., 2019). High levels of BDNF are associated with neuronal protection (Almeida et al., 2005), while low levels have been associated with normal aging and pathological conditions such as Alzheimer's disease

and Parkinson's disease (Bathina and Das, 2015; Miranda et al., 2019). Furthermore, BDNF is involved in the regulation of energy balance, and may act as an anorexigenic signaling molecule (Liu et al., 2014; Rios et al., n.d.). As such, dysregulated levels of BDNF have been associated with obesity (Genzer et al., 2016). In our study, DIO led to a 2-fold increase in BDNF mRNA expression in B6J females in the olfactory bulb (Figure 4.12). Previous studies in rodent models have also shown an upregulation of BDNF mRNA or protein due to DIO, however, these results were in males and in different brain regions. For example, a study using male B6 mice fed a HFD for eight weeks found that BDNF mRNA and protein was upregulated in whole brain tissue and in HT-4 hippocampal neurons (Genzer et al., 2016). In a study using male Long-Evans rats fed a HFD for 72 hours, the mRNA expression of BDNF was upregulated in the hippocampus (Gan et al., 2015). In contrast, a study using female Fisher 344 rats fed a diet high in fat and sugar found that BDNF protein and mRNA expression in the hippocampus was reduced after 6 months of diet treatment (Molteni et al., 2002). In our mice, we found no effect of DIO on hippocampal BDNF gene expression. However, there was a significant sex effect in the D2J strain, with D2J female mice expressing 2.5-fold higher levels of BDNF mRNA compared to D2J males (Figure 4.11). It has been reported previously that female rats express higher levels of BDNF in the hippocampus and cortex (Chan and Ye, 2017). A study using Long-Evans male and female rats fed a control diet or HFD for four days and four weeks compared BDNF mRNA expression in the ventromedial nucleus of the hypothalamus (Liu et al., 2014). At both time points, the

expression of BDNF was higher in females compared to males, regardless of diet. In a study with male and female Wistar rats exposed to an enriched environment, females expressed higher levels of BDNF protein compared to males in both the control and enrichment-treated groups (Bakos et al., 2009). The D2J strain in our study follow a similar pattern of increased hippocampal BDNF expression in females, however we did not observe the same effect in the B6J strain. To gain a better understanding of these sex- and strain-dependent gene expression trends in different brain regions, future investigations could include an evaluation of BDNF expression levels for both mRNA and protein in several brain regions or subregions. To the best of our knowledge, our study is the first study to reveal DIO-induced alterations in BDNF mRNA expression in the murine olfactory bulb. As BDNF has an important role in neural plasticity and neuronal protection in several brain regions, including the olfactory bulb, this could have important health implications regarding the development of behavior disorders and neurodegenerative disease.

Conclusion

In conclusion, the results from this study provide evidence that the impact of DIO on behavior, dopamine biology, and gene expression is influenced by sex and strain. Behavior evaluations showed that the B6J male mice fed a HFD were impacted the most through their display of reduced TDT throughout the entire open field test, reduced rate of habituation to a novel environment, lack of motivation to shred, and elevated anxiety levels in the open field. Furthermore, DRD2 was upregulated only in male B6J mice in

the striatum due to DIO. These results confirmed our hypothesis that male mice would be impacted more than females, and that the B6J strain would be less resistant to the effects of DIO compared to the D2J strain. We did find, however, that dopamine biology alterations and gene expression dysregulation due to DIO were present in females and the D2J strain. Dopamine clearance in the dorsal striatum was significantly reduced in both male and female D2J mice due to DIO, while in the NAc core, reductions in dopamine clearance occurred for male mice of both strains. Our evaluation of mRNA gene expression demonstrated that DRD2 in the striatum and olfactory bulb and TH in the olfactory bulb were only dysregulated in males due to DIO. BDNF mRNA expression, however, was only impacted by DIO in B6J females in the olfactory bulb. The major strengths of this study were the inclusion of two strains and both sexes for the evaluation of sex and genetic influences on DIO impact in the brain, the use of multiple performance tests to assess the impact of DIO on a variety of behaviors, and the use of real time ex-vivo fast scan cyclic voltammetry to evaluate dopamine biology. Moreover, we have provided novel information regarding the neurobiological impact of DIO on the olfactory bulb, a brain region that previously has not been investigated in the context of DIO with sex and strain influences. In terms of study limitations, we measured mRNA expression exclusively, but not protein expression due to lack of tissue availability. Furthermore, while we carefully planned our behavior tests and tissue collection schedule to limit variability due circadian variation and estrous cycle, we could not perform all measurements at the same time of day due to the number of mice involved.

To account for this, we used a Latin square technique for all measurements and procedures to counterbalance potential variations that could impact our data. In summary, our study provides evidence for important sex and strain influences on the impact of DIO-induced behavior alterations and neurobiology dysregulation. As the incidence of obesity continues to rise worldwide, these data have key health implications related to debilitating behavior disorders. The development of treatment and rehabilitation programs should therefore account for these apparent neurobiological differences in sex and genetics.

CHAPTER V

EPILOGUE

The overarching goal of this research was to identify sex and strain influences on neurobiological and behavioral changes due to DIO. In our first study (Chapter III), we investigated main effects and interactions between diet, sex, and strain on Fe, Mn, Cu, and Zn homeostasis and mRNA gene expression for proteins related to trace element metabolism and neurodegeneration. In our second study (Chapter IV), we examined main effects and interactions between diet, sex, and strain on behavior, dopamine release and reuptake, and mRNA gene expression for proteins related to behavior and dopamine biology. These studies revealed a heterogeneous effect of DIO on many of the neurobiological variables that we evaluated. For example, in the striatum, Fe was significantly elevated in B6J female mice but not male mice due to DIO. Similarly, in the hippocampus, Zn was increased in D2J males but decreased in D2J females. There was also a dramatic induction of DMT1, alpha synuclein, and APP in this brain region due to DIO, but only in the B6J males. Behavior assessments demonstrated that B6J male mice fed a HFD were impacted the most through their display of significantly reduced locomotion, reduced rate of habituation, lack of motivation, and elevated anxiety levels. Interestingly, these mice also showed a significant upregulation of DRD2. Dopamine clearance in the dorsal striatum was significantly reduced in both male and female D2J

mice due to DIO, while in the NAc core, reductions in dopamine clearance occurred for male mice of both strains fed HFD.

In order to establish an effective DIO model in rodents, it is important to select appropriate diets and mouse strains. Our experimental design included male and female B6J and D2J mouse strains which were randomly assigned to a LFD (10% kcal fat) or HFD (60% kcal lard-based fat) for 16 weeks. These strains have been shown to respond efficiently to a HFD to create the DIO state (Alexander et al., 2006; Montgomery et al., 2013; West et al., 1992). Our data corroborates with these reports, as males and females from both the B6J and D2J strains fed a HFD had significantly higher body weight and greater volume of adipose tissue at the end of the diet treatment. There are other reports in the literature, however, that show D2J mice respond less efficiently to a HFD, including lack of weight gain (Kirk et al., 1995), delayed weight gain (Norris et al., 2016), or similar weight gain compared to a control LFD (Funkat et al., 2004). These studies either initiated their diet treatments later than three weeks of age (typically 8-10 weeks old), or used a fat source other than lard in their HFD. Using a diet with 60% lard as the source of fat and initiating our diet at approximately three weeks old provided a quick and effective DIO model for our study. It should be noted that other diets, such as a 45% HFD or a cafeteria style diet may mimic the human HFD more closely, however, the 60% HFD is widely used in animal research and produces physiological responses that are relevant to human physiology (Lutz and Woods, 2012).

A limitation of our study is that our experimental design did not allow for the partitioning of effects of DIO versus HFD. Instead, we can conclude that the impact we observed on trace element homeostasis, gene expression, etc., was due to a state of DIO induced by a high saturated fat diet, or restated, a combination of HFD and DIO. To determine if the neurobiological and behavioral changes were due to components of the HFD and not a state of DIO, a future experimental design using a LFD and HFD treatment could incorporate a mouse strain that is resistant to weight gain, such as the AJ or Mbd2 ^{-/-} strains (Cheng et al., 2016; Surwit et al., 1995). Furthermore, there are alternative methods to induce dietary obesity, such as a 45% HFD, cafeteria style diet, or high fat diets containing different unsaturated and saturated fatty acid blends (Barrett et al., 2016; Speakman, 2019). Future experiments could utilize these different diets to provide a comparison of DIO states with different origins.

Reviewing our findings from both studies, it is interesting that B6J male mice displayed significant behavior changes, increased Fe and Mn in the olfactory bulb, and dysregulation of mRNA expression of genes related to neurodegeneration. These mice had significantly reduced locomotion throughout the open field test, decreased motivation in the nestlet shredding test, and increased anxiety-like behavior assessed by fecal boli quantity. Furthermore, B6J males had significantly reduced habituation, a measure of learning and memory, which could indicate hippocampal impairment. It is possible that these behavior changes are related to the dysregulated mRNA expression that occurred in B6J males due to DIO. For example, there was a substantial induction

of DMT1, alpha synuclein, and APP in the hippocampus for B6J males, and an upregulation of DRD2 in the striatum. The induction of alpha synuclein and APP could be a sign of early neurodegeneration, which could manifest as reduced habituation rate. A follow up experiment should include protein testing for alpha synuclein and APP in the hippocampus. Furthermore, an evaluation of oxidative stress, such as the F2-isoprostane test for lipid peroxidation, should be performed in the hippocampus to test for signs of neurodegeneration. The upregulation of DRD2 in the striatum indicates dysregulation of dopamine biology, which may be associated with the reduction of motor activity in the open field and lack of motivation to shred. Moreover, B6J males had a reduced rate of dopamine reuptake in the NAc core, indicating a disruption of the reward circuitry in these mice. Future work with B6J mice should include behavior testing using the elevated plus maze, which may be a more accurate test for anxiety compared to center entries in an open field. The nestlet test for motivation could be modified to extend the time from 30 minutes to 1-2 hours, which may provide a better determination of latency to shred. Additionally, this test can be recorded to monitor other behaviors such as locomotion, grooming, and rearing. Since the nestlet shredding test is relatively easy to set up and provides a quantitative measurement to reduce subjectivity, this test is recommended for future behavior assessments. The open field test also provides several useful behavior assessments; however, our current apparatus is not large enough for the obese mice. Most labs use a 40" x 40" surface area made of opaque plastic for easy cleaning. Our surface measures 29" by 29" and is lined with

paper. Although the paper creates an opaque arena, the mice can dig into the paper, which occurred often with the D2J strain. Furthermore, the sides of our testing cubes displayed a reflection, which could impact the behavior of the mice. Nevertheless, our findings demonstrate that B6J male mice are a good model for evaluating the effects of DIO on behavior change and neurobiological alterations.

In Chapters III and IV, our results demonstrate that D2J females have a unique response to DIO. D2J females fed a HFD were the only group to display a decrease in Zn in the hippocampus and the only group to have a negative discrimination index in the learning and memory test. For the assessment of correlations with body weight, the D2J females had a positive correlation between weight and Fe and weight and DMT1 expression in the striatum. In the hippocampus, D2J females showed positive correlations between weight and alpha synuclein and weight with APP expression, and showed a negative correlation with weight and Zn. Furthermore, the D2J group had a negative correlation between weight and TDT and weight with velocity. These correlations are useful for understanding relationships between weight and neurobiological changes, and provide evidence that excess body weight could be a direct cause of these alterations. Although other treatment groups appeared to have statistically significant correlations between body weight and different neurobiological variables, the data were often clustered and could not be characterized as a linear relationship. The Zn reduction in the hippocampus of female D2J mice with the negative discrimination index for learning and memory could be investigated further in future

studies. It is possible that dysregulated Zn due to DIO is associated with reductions in cognition. There are alternative methods for evaluating memory and cognition that may provide a better understanding of the effects of DIO on the hippocampus, including the Morris water maze test (Cordner and Tamashiro, 2015). Moreover, the novel object test can be modified to change the spatial arrangement of objects rather than introducing a new object, which has also been shown to reflect neurobiological changes in the hippocampus (Cordner and Tamashiro, 2015). Future work could also include an evaluation of the prefrontal cortex for trace element dysregulation and gene expression alterations, as this brain region is also involved in memory, learning, and cognition (Cordner and Tamashiro, 2015). Furthermore, the impact of DIO on gene and protein expression for ZIP and ZRT transporters could be investigated in the hippocampus and prefrontal cortex to explore potential links to dysregulated Zn in the brain.

The olfactory bulb is a brain region that has not been studied extensively in the context of DIO. A recent study in Parkinson's disease patients found that Fe was elevated in this brain region (Gardner et al., 2017). In a study using brain tissue from Alzheimer's disease patients, both Fe and Zn were increased in the olfactory bulb (Samudralwar et al., 1995). Another study reported alpha synuclein upregulation in the olfactory bulb in the early development of Parkinson's disease (Adler and Beach, 2016). In our lab, we found previously that DIO in male B6J mice can lead to upregulated alpha synuclein and increased Fe in the midbrain, both of which are hallmarks of neurodegeneration (Han et al., 2019). Based on these studies, we were interested in

exploring the effects of DIO on olfactory bulb neurobiology. We found a significant increase in Fe and Mn in B6J male mice fed a HFD, but no increases in Zn in this brain region. It is interesting that the dysregulation of trace elements in the olfactory bulb only impacted the male B6J mice. In contrast, the impact of DIO on gene expression alterations in the olfactory bulb predominantly affected D2J males. D2J males fed a HFD exhibited significantly upregulated DMT1, alpha synuclein, APP, DRD2, and TH in this brain region. The only other treatment group that was also affected was the B6J females with an upregulation of BDNF in the olfactory bulb for mice fed a HFD. These studies provide evidence that the olfactory bulb is a brain region that is impacted by a state of DIO in sex and strain dependent manner. The olfactory bulb is relatively easy to separate from the rest of the brain during the dissection process, making it simple to isolate and store for future DIO studies. Future work could include an evaluation of DIO-associated trace element dysregulation and gene expression alteration over time to study the effects of DIO on aging in this brain region. Since there were clear differences in neurobiological disturbances between males and females and between the B6J and D2J strain, both sexes of these strains could be used for future work. However, since female mice appeared to be more resistant to DIO-associated alterations in the olfactory bulb, it may be justified to use only males for the comparison of strain differences.

In our study of gene expression, we selected the gene that was recommended for best coverage to obtain a broad scope understanding of each gene. However, this

does not differentiate between different isoforms that may exist for each gene. Future work in our lab may include an assessment of DIO-associated alterations of specific isoforms. For example, DRD2 has two isoforms, D2L and D2S, with the long D2L predominantly expressed in the brain (Baik, 2013). Recent studies in humans and mice show that these isoforms are differentially expressed in addiction studies (Moyer et al., 2011) and may have different functions *in vivo* (Usiello et al., 2000; Wang et al., 2000). DMT1 has four known isoforms: 1A/(+IRE), 1A/(-IRE), 1B/(+IRE), 1B/(-IRE) that are expressed at different levels (Skjørringe et al., 2015), however, the trace element transport function of all four isoforms is similar (Mackenzie et al., 2007). Nevertheless, a more comprehensive examination of DMT1 gene expression based on specific isoforms may provide a better understanding of the specific mechanisms involved in obesity-induced gene expression alteration. Furthermore, future studies with IRP in the brain should include IRP2, which has been found recently to have a critical impact on brain iron metabolism (Zhang et al., 2014), neurodegeneration (Ghosh et al., 2015), and behavior (Zumbrennen-Bullough et al., 2014). A case study performed on a 16-year-old male with evidence of neurodegeneration was found to lack the gene coding for IRP2 (Costain et al., 2019), and is the first study to show IRP2 dysregulation in the human brain.

In summary, our findings provide evidence for important sex and strain differences on the impact of DIO-associated behavior alterations and neurobiology dysregulation. As the incidence of obesity continues to rise worldwide, these findings

have key health implications related to debilitating behavior disorders and the development of neurodegenerative disease that can be triggered by an energy dense diet and a state of DIO. Future targeted therapies for obesity, behavior impediments, and brain diseases may need to be specifically tailored to account for sex and genetics as key biological factors.

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APPENDIX A

SUPPLEMENTARY DATA

Supplementary Table A1. Rodent Diets D12450J and D12492 from Research Diets, Inc.

Ingredient	D12450J (10% kcal from fat)		D12492 (60% kcal from fat)	
	Grams	Kilocalories	Grams	Kilocalories
Casein, Lactic, 30 mesh	200.00	800.00	200.00	800.00
L-Cysteine	3.00	12.00	3.00	12.00
Cornstarch	506.20	2024.80	-	-
Lodex 10	125.00	500.00	125.00	500.00
Fine granulated sucrose	72.80	291.20	72.80	291.20
Solka Floc fiber, FCC200	50.00	200.00	50.00	200.00
Soybean oil, USP	25.00	225.00	25.00	225.00
Lard	20.00	180.00	245.00	2205.00
Mineral mix S10026B	50.00	-	50.00	-
Choline bitartrate	2.00	-	2.00	-
Vitamin mix V10001C	1.00	-	1.00	-
Yellow dye FD&C #5	0.04	-	-	-
Blue dye FD&C #1	0.01	-	0.05	-
Total	1055.05	4233.00	773.85	4233.20
Caloric Summary				
Fat		10% kcal		60% kcal
Carbohydrate		70% kcal		20% kcal
Protein		20% kcal		20% kcal
Energy Density		3.82 kcal/g		5.21 kcal/g

A description of diets used for the 16-week treatment. D12450J (10% kcal from fat) represents the LFD and D12492 (60% kcal from fat) represents the HFD. Saturated fat in the form of lard is the major source of fat in this study.

Supplementary Table A2. Trace element Concentrations in Specific Brain Regions of Male and Female B6J and D2J Mice

Brain Region	Treatment Group	Iron	Manganese	Copper	Zinc
Hippocampus	BML	373 ± 3	6.82 ± 0.48	118.5 ± 1.3	367 ± 32
	BMH	431 ± 32	7.28 ± 0.41	134.3 ± 11.3	409 ± 75
	BFL	435 ± 22	7.00 ± 0.58	119.4 ± 16.6	350 ± 27
	BFH	470 ± 35	8.81 ± 1.19	176.7 ± 20.4	473 ± 59
	DML	357 ± 24	7.41 ± 0.96	122.1 ± 6.3	338 ± 36
	DMH	386 ± 24	6.95 ± 0.81	118.3 ± 15.8	549 ± 94
	DFL	452 ± 34	8.03 ± 0.59	157.0 ± 8.3	581 ± 73
	DFH	405 ± 36	6.76 ± 0.83	131.6 ± 12.6	370 ± 11
Midbrain	BML	479 ± 96	10.00 ± 0.96	109.0 ± 17.0	400 ± 95
	BMH	369 ± 34	7.72 ± 0.69	82.0 ± 7.2	251 ± 27
	BFL	444 ± 24	10.58 ± 1.09	98.4 ± 5.5	340 ± 11
	BFH	443 ± 49	9.43 ± 1.46	88.5 ± 10.0	314 ± 49
	DML	551 ± 85	12.59 ± 1.83	125.2 ± 16.5	543 ± 118
	DMH	470 ± 83	11.53 ± 2.27	76.9 ± 3.0	403 ± 118
	DFL	473 ± 105	12.58 ± 1.49	124.8 ± 13.5	407 ± 81
	DFH	402 ± 38	11.49 ± 1.30	85.6 ± 11.9	379 ± 103
Olfactory Bulb	BML	572 ± 66	12.33 ± 0.28	102.3 ± 8.3	223 ± 26
	BMH	1252 ± 282	20.45 ± 4.49	118.3 ± 7.5	275 ± 30
	BFL	931 ± 31	18.15 ± 1.84	126.1 ± 10.0	278 ± 19
	BFH	818 ± 116	16.66 ± 2.02	116.2 ± 17.1	209 ± 11
	DML	758 ± 82	16.78 ± 1.89	127.0 ± 14.6	306 ± 60
	DMH	844 ± 145	14.99 ± 1.90	108.0 ± 8.4	236 ± 29
	DFL	887 ± 92	16.76 ± 1.82	113.7 ± 9.8	281 ± 36
	DFH	885 ± 88	14.45 ± 1.25	121.4 ± 15.3	334 ± 44
Striatum	BML	448 ± 63	10.15 ± 1.04	129.8 ± 17.5	712 ± 157
	BMH	409 ± 9	9.44 ± 0.45	97.1 ± 0.8	710 ± 141
	BFL	454 ± 40	8.44 ± 0.70	107.4 ± 12.5	716 ± 175
	BFH	598 ± 48	10.32 ± 1.30	111.4 ± 28.5	563 ± 100
	DML	381 ± 35	8.04 ± 0.55	94.5 ± 8.8	696 ± 185
	DMH	406 ± 6	8.24 ± 0.45	93.1 ± 2.1	569 ± 46
	DFL	335 ± 32	9.51 ± 0.73	95.6 ± 11.2	731 ± 113
	DFH	410 ± 39	9.58 ± 0.67	123.7 ± 11.5	552 ± 54

Data are represented as mean ± SEM in units of µg trace element/g protein. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet

Supplementary Table A3. Striatum mRNA Gene Expression Δ Ct Values

Treatment Group	DMT1	IRP1	Cp	aSyn	D2	TH
BML	17.37 \pm 0.06	18.37 \pm 0.13	18.44 \pm 0.27	9.81 \pm 0.12	20.71 \pm 0.10	16.75 \pm 0.32
BMH	17.47 \pm 0.08	18.36 \pm 0.15	18.20 \pm 0.24	10.07 \pm 0.15	19.68 \pm 0.41	16.36 \pm 0.18
BFL	17.00 \pm 0.21	18.06 \pm 0.23	18.09 \pm 0.12	14.07 \pm 0.14	18.47 \pm 0.28	16.69 \pm 0.38
BFH	17.22 \pm 0.07	18.14 \pm 0.05	17.92 \pm 0.18	14.42 \pm 0.07	18.34 \pm 0.42	16.43 \pm 0.20
DML	17.43 \pm 0.14	18.20 \pm 0.16	18.07 \pm 0.13	14.38 \pm 0.14	16.67 \pm 0.57	19.03 \pm 0.41
DMH	17.36 \pm 0.15	18.09 \pm 0.09	18.10 \pm 0.08	14.31 \pm 0.19	16.64 \pm 0.30	19.33 \pm 0.34
DFL	17.36 \pm 0.02	18.14 \pm 0.08	19.00 \pm 0.16	14.32 \pm 0.21	17.72 \pm 0.07	19.57 \pm 0.25
DFH	17.17 \pm 0.04	18.09 \pm 0.06	18.38 \pm 0.16	14.43 \pm 0.03	17.26 \pm 0.15	19.72 \pm 0.32

Data are represented as mean \pm SEM. Δ Ct values were normalized using the endogenous control, 18S. Higher Δ Ct values indicate lower levels of gene expression. The Δ Ct values reported here were used to determine fold change in gene expression when comparing LFD (control) to HFD or male (control) to female using the comparative Ct method. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet. DMT1=divalent metal transporter 1, IRP1=iron regulatory protein 1, Cp=ceruloplasmin, aSyn=alpha synuclein, D2 = dopamine receptor D2, TH = tyrosine hydroxylase.

Supplementary Table A4. Hippocampus mRNA gene expression Δ Ct values

Treatment Group	DMT1	Cp	CTR1	aSyn	APP	BDNF
BML	16.79 \pm 0.21	12.91 \pm 0.08	18.49 \pm 0.17	13.50 \pm 0.32	10.55 \pm 0.41	19.12 \pm 0.28
BMH	14.56 \pm 0.45	13.61 \pm 0.20	18.56 \pm 0.18	10.74 \pm 0.40	7.30 \pm 0.37	18.89 \pm 0.26
BFL	17.51 \pm 0.06	18.19 \pm 0.23	18.18 \pm 0.36	15.04 \pm 0.39	8.51 \pm 0.11	19.11 \pm 0.26
BFH	17.42 \pm 0.12	18.05 \pm 0.20	18.10 \pm 0.22	14.25 \pm 0.08	8.09 \pm 0.11	18.67 \pm 0.08
DML	16.96 \pm 0.12	17.86 \pm 0.19	18.23 \pm 0.25	14.34 \pm 0.35	10.86 \pm 0.21	19.16 \pm 0.17
DMH	17.24 \pm 0.07	17.51 \pm 0.23	18.27 \pm 0.10	14.14 \pm 0.31	11.03 \pm 0.10	19.39 \pm 0.43
DFL	17.18 \pm 0.16	18.07 \pm 0.19	19.56 \pm 0.22	13.98 \pm 0.05	11.10 \pm 0.13	17.92 \pm 0.18
DFH	17.02 \pm 0.10	18.02 \pm 0.14	19.20 \pm 0.27	13.75 \pm 0.90	10.76 \pm 0.08	17.95 \pm 0.13

Data are represented as mean \pm SEM. Δ Ct values were normalized using the endogenous control, 18S. Higher Δ Ct values indicate lower levels of gene expression. The Δ Ct values reported here were used to determine fold change in gene expression when comparing LFD (control) to HFD or male (control) to female using the comparative Ct method. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet. DMT1=divalent metal transporter 1, Cp=ceruloplasmin, CTR1=copper transporter protein 1, aSyn=alpha synuclein, APP = amyloid precursor protein, BDNF = brain-derived neurotrophic factor.

Supplementary Table A5. Olfactory Bulb mRNA Gene Expression Δ Ct Values

Treatment Group	DMT1	aSyn	APP	BDNF	D2	TH
BML	14.01 \pm 0.20	13.40 \pm 0.03	11.01 \pm 0.23	19.04 \pm 0.35	19.55 \pm 0.16	14.70 \pm 0.74
BMH	14.40 \pm 0.20	13.60 \pm 0.04	10.97 \pm 0.21	19.01 \pm 0.17	20.84 \pm 0.46	15.45 \pm 0.55
BFL	12.82 \pm 0.36	12.25 \pm 0.31	11.40 \pm 0.34	19.82 \pm 0.28	18.77 \pm 0.50	13.82 \pm 0.25
BFH	12.79 \pm 0.40	12.50 \pm 0.25	11.68 \pm 0.40	18.87 \pm 0.35	19.86 \pm 0.69	14.41 \pm 0.47
DML	18.23 \pm 0.05	14.47 \pm 0.21	8.89 \pm 0.03	19.92 \pm 0.19	21.28 \pm 0.24	16.34 \pm 0.33
DMH	17.27 \pm 0.34	13.83 \pm 0.02	7.54 \pm 0.29	20.04 \pm 0.24	19.68 \pm 0.48	14.67 \pm 0.66
DFL	17.85 \pm 0.38	14.38 \pm 0.23	12.27 \pm 0.44	20.55 \pm 0.20	20.74 \pm 0.25	15.80 \pm 0.15
DFH	17.79 \pm 0.15	14.26 \pm 0.07	12.62 \pm 0.38	20.11 \pm 0.11	20.62 \pm 0.33	15.27 \pm 0.19

Data are represented as mean \pm SEM. Δ Ct values were normalized using the endogenous control, 18S. Higher Δ Ct values indicate lower levels of gene expression. The Δ Ct values reported here were used to determine fold change in gene expression when comparing LFD (control) to HFD or male (control) to female using the comparative Ct method. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet. DMT1=divalent metal transporter 1, aSyn=alpha synuclein, APP = amyloid precursor protein, BDNF = brain-derived neurotrophic factor, D2 = dopamine receptor D2, TH = tyrosine hydroxylase.

Supplementary Table A6. Midbrain mRNA Gene Expression Δ Ct Values

Treatment Group	aSyn	Cp	TH
BML	15.34 \pm 0.32	18.52 \pm 0.27	18.32 \pm 1.44
BMH	15.19 \pm 1.14	18.24 \pm 0.59	17.95 \pm 1.42
DML	13.76 \pm 1.48	17.52 \pm 0.20	16.66 \pm 0.22
DMH	12.83 \pm 0.46	17.13 \pm 0.59	17.30 \pm 1.36

Data are represented as mean \pm SEM. Δ Ct values were normalized using the endogenous control, 18S. Higher Δ Ct values indicate lower levels of gene expression. The Δ Ct values reported here were used to determine fold change in gene expression when comparing LFD (control) to HFD or male (control) to female using the comparative Ct method. Treatment group letter codes: B=B6J, D=D2J, M=male, L=low fat diet, H=high fat diet. aSyn=alpha synuclein, Cp=ceruloplasmin, TH = tyrosine hydroxylase.

Supplementary Table A7. Total Distance Traveled for Male and Female B6J and D2J Mice in the Open Field

Treatment Group	Total Distance Traveled (m) per Five-min Timeframe					
	0-5	5-10	10-15	15-20	20-25	25-30
BML	18.1 ± 1.2	12.3 ± 0.9	11.6 ± 0.4	10.4 ± 0.5	11.3 ± 0.8	9.7 ± 0.7
BMH	11.2 ± 0.8	8.3 ± 0.6	8.0 ± 0.5	7.9 ± 0.5	6.7 ± 0.3	6.5 ± 0.3
BFL	15.7 ± 1.2	11.8 ± 0.7	11.1 ± 0.6	12.3 ± 0.5	10.9 ± 0.6	10.9 ± 0.8
BFH	12.9 ± 0.7	9.3 ± 0.6	8.8 ± 0.6	9.6 ± 0.7	8.7 ± 0.9	8.6 ± 0.8
DML	8.7 ± 1.2	9.9 ± 1.4	10.4 ± 0.8	9.8 ± 0.8	7.6 ± 1.2	6.8 ± 1.1
DMH	5.9 ± 0.6	7.5 ± 0.8	6.3 ± 1.0	4.9 ± 0.7	4.1 ± 0.7	4.2 ± 1.0
DFL	7.8 ± 0.5	10.2 ± 1.7	7.3 ± 0.7	5.3 ± 1.0	4.5 ± 0.5	6.6 ± 1.3
DFH	7.1 ± 0.9	7.1 ± 1.0	5.2 ± 0.6	6.9 ± 1.1	3.1 ± 0.4	3.3 ± 0.6

Data are represented as mean ± SEM. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet.

Supplementary Table A8. Velocity for Male and Female B6J and D2J Mice in the Open Field

Treatment Group	Velocity (mm/s) per Five-min Timeframe					
	0-5	5-10	10-15	15-20	20-25	25-30
BML	60.4 ± 3.8	40.6 ± 3.4	38.6 ± 1.3	35.1 ± 1.8	38.0 ± 2.6	32.6 ± 2.4
BMH	37.4 ± 2.8	27.9 ± 2.2	25.9 ± 1.5	26.6 ± 1.7	22.4 ± 1.0	21.7 ± 0.9
BFL	52.7 ± 4.1	39.4 ± 2.2	37.0 ± 1.8	41.1 ± 1.5	36.3 ± 1.8	36.0 ± 2.8
BFH	43.3 ± 2.6	31.1 ± 2.1	29.2 ± 2.1	31.9 ± 2.4	29.2 ± 2.8	29.0 ± 2.6
DML	25.8 ± 2.7	29.9 ± 4.4	33.1 ± 2.6	31.5 ± 2.8	25.0 ± 4.6	18.0 ± 3.6
DMH	19.3 ± 2.0	25.1 ± 2.7	20.9 ± 3.4	16.6 ± 2.1	13.8 ± 2.5	13.9 ± 3.3
DFL	26.0 ± 1.7	34.3 ± 5.8	24.4 ± 2.2	17.9 ± 3.2	15.1 ± 1.6	21.9 ± 4.4
DFH	23.8 ± 2.9	23.6 ± 3.4	17.4 ± 2.0	21.4 ± 4.6	10.2 ± 1.4	10.8 ± 2.1

Data are represented as mean ± SEM. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet.

Supplementary Table A9. Behavior Tests for Exploration, Anxiety, Compulsivity, Motivation, and Memory

Treatment Group	Habituation	Fecal Boli	Center Entries	Nestlet Shredding	Novel Object DI
BML	263 ± 32	5.33 ± 0.75	25.2 ± 1.6	2.38 ± 0.76	0.057 ± 0.068
BMH	163 ± 25	7.78 ± 0.60	22.4 ± 1.8	0.11 ± 0.04	0.146 ± 0.067
BFL	152 ± 32	4.11 ± 0.51	22.2 ± 1.6	0.31 ± 0.14	0.090 ± 0.055
BFH	127 ± 31	4.67 ± 0.76	19.3 ± 1.3	0.61 ± 0.20	0.166 ± 0.069
DML	156 ± 45	5.89 ± 0.72	7.9 ± 2.1	3.00 ± 0.74	0.098 ± 0.087
DMH	101 ± 18	5.38 ± 0.78	9.8 ± 3.3	2.50 ± 0.63	0.101 ± 0.179
DFL	142 ± 41	5.33 ± 1.03	10.7 ± 3.0	3.73 ± 0.92	0.081 ± 0.136
DFH	169 ± 27	5.44 ± 0.63	6.6 ± 2.2	1.62 ± 0.48	-0.12 ± 0.098

Habituation measures exploration and memory with learning. Fecal boli and center entries measure anxiety. Nestlet shredding measures compulsivity, motivation, and welfare. Novel Object Discrimination Index measures learning and memory. Data are represented as mean ± SEM. For the center entries test, data from the first five-minute segment in the open field were used for the B6J mice analysis, and data from the second five-minute segment was used for the D2J analysis. These time frames were selected based on greatest total distance travelled for each strain. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet. DI = discrimination index.

Supplementary Table A10. Dopamine Release and Reuptake in the Striatum

Treatment Group	Dopamine Release DS	Dopamine Reuptake DS	Dopamine Release VS	Dopamine Reuptake VS
BML	151.5 ± 18.3	3.22 ± 0.32	174.0 ± 11.6	2.89 ± 0.32
BMH	136.8 ± 8.5	3.73 ± 0.36	180.8 ± 13.6	1.63 ± 0.19
BFL	128.3 ± 6.1	4.10 ± 0.43	185.8 ± 10.7	2.23 ± 0.33
BFH	163.6 ± 9.3	3.89 ± 0.46	187.2 ± 7.0	2.57 ± 0.63
DML	133.7 ± 6.7	4.81 ± 0.56	196.5 ± 11.5	3.53 ± 0.43
DMH	149.0 ± 8.7	3.41 ± 0.35	206.8 ± 8.7	2.13 ± 0.24
DFL	140.0 ± 7.8	4.74 ± 0.64	188.0 ± 26.1	3.64 ± 0.58
DFH	145.9 ± 7.3	3.44 ± 0.32	214.1 ± 6.8	3.12 ± 0.24

Data are represented as mean ± SEM. Dopamine release was calculated as a ratio of five-pulse release to one-pulse release to provide information regarding the dynamic capacity to release dopamine from the dorsal and ventral striatum. Units for dopamine release are % pulse. Dopamine reuptake is measured as maximal rate of dopamine uptake (Vmax) by dopamine transporter. Units for reuptake are μmol/L·s. DS = dorsal striatum, VS = ventral striatum. Treatment group letter codes: B=B6J, D=D2J, M=male, F=female, L=low fat diet, H=high fat diet.

APPENDIX B

BEHAVIOR TESTING



Figure B1. Open Field Set Up. Four acrylic cubes were used for the open field and novel object recognition tests with one mouse placed in each cube. Each cube is assigned an individual camera with recording software. Four mice were evaluated at one time for each round of testing.



Figure B2. Open Field Single Cube. Each acrylic cube measures 29 cm x 29 cm x 38 cm. Mice were placed at the center of the cube at the start of each experiment.



Figure B3. Objects Used for Novel Object Recognition Test. Nonporous objects were chosen with similar size and color but distinct shape. Two owls were used in phase II (familiarization phase). One owl and one squirrel were used for phase III (testing phase), with the owl as the familiar object and the squirrel as the new object.

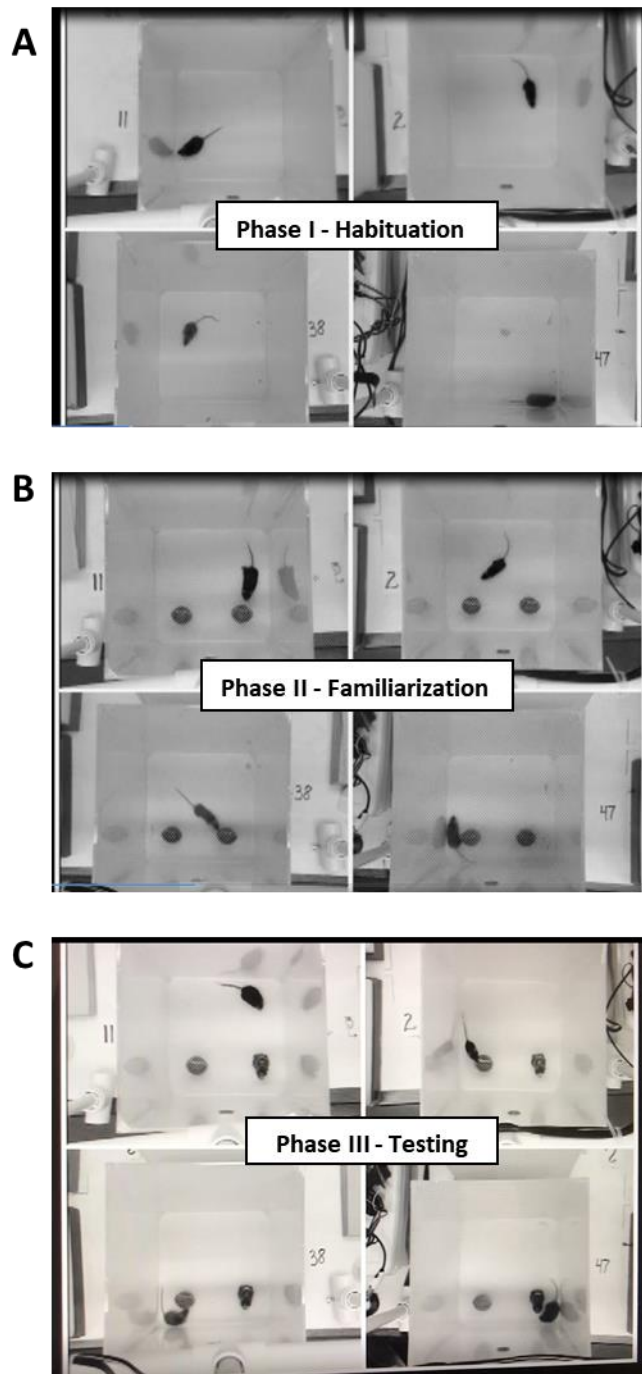


Figure B4. Novel Object Recognition Test. Phase I (A) is the 30-minute habituation phase for mice to gain exposure to the testing arena. Phase II (B) is the familiarization phase for mice to explore an identical object for five minutes. Phase III (C) is the testing phase for mice to explore either the familiar object or new object for five minutes. Phase III occurs two hours after phase II and is used to evaluate memory.



Figure B5. Nestlet Shredding Set Up. Polycarbonate mouse cages were filled with bedding to a depth of 0.5 cm. Pre-weighed cotton nestlet squares (5.8cm x 5.8cm x 0.2cm) were placed in the middle of each cage before placing each mouse inside. Fitted filter tops were used to enclose the cage during testing (tops and nestlet squares not shown here).



Figure B6. Nestlet Shredding Result Example. After each 30-minute nestlet shredding test, shredded nestlet material was carefully removed from each nestlet square. Individual nestlets were dried for 24 hours before weighing. Percent shredding was calculated for each mouse as a measure of motivation and compulsivity.